EFFECTS OF ANTICANCER AGENTS ON GLUCOSE TRANSPORT IN L929 AND EHRLICH ASCITES TUMOR CELLS

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CONTENTS

ABSTRACT

Methotrexate (MTX) suppressed the growth of Ehrlich ascites tumour (EAT) cells *in vivo*. The rate of cellular uptake of glucose and the number of glucose carriers, as determined by cytochalasin B binding method, were also found to be reduced. Administration of thymidine could partially reverse the anti-tumour effect of methotrexate and simultaneously this treatment could partially reverse the reduction of the number of glucose carriers on tumour cells by methotrexate. However, the administration of uridine exhibited no effect on tumour cells.

The interferon inducers, polyriboinosinic : polyribocytidylic acid (poly I:C), statolon and tilorone were found significantly effective in arresting the Ehrlich ascites tumor growth and also the inhibition of glucose transport activity. Another interferon inducer, 2-aminoethylisothiouronium bromide (AET), was found ineffective in suppressing tumour growth and at the same time it was also ineffective in reducing the glucose transport rate and the number of glucose carrier. Intraperitoneal administration of inducers was found having higher efficacy against tumor growth than other administration routes. Pretreatment of poly I:C was also effective in prevention of tumor growth and suppression of glucose carrier number of tumour cells. The rather low titer of serum interferon measured 24 hours after inducers administration suggests that the inducers might also exert direct or indirect

effects, besides through interferon, on the tumour cells.

Tumor necrosis factor (TNF) were cytotoxic to L929 cells *in vitro*. [^{3}H]-thymidine release from the cells was evident as early as 12 hours after incubation with TNS. *C. parvum*-induced TNS suppressed EAT growth and inhibited the glucose uptake rate *in vitro*. In contrast, this TNS preparation increased the glucose uptake rate of cultured L929 cells. TNS preparations induced by zymosan and *Listeria monocytogenes* could also increase glucose uptake rate of cultured L929 cells. The nature of cytochalasin B binding sites of L929 cells differed from that of EAT cells. The binding sites on L929 cells were not glucose-reversible. Besides increasing glucose uptake, TNS-treated L929 cells exhibited increases in Ca^{2+} uptake and uridine diffusion rate while the methionine and leucine uptake remained relatively unchanged.

Studies of glucose transport system of EAT cells during methotrexate, interferon inducers and TNS treatments in the present thesis indicated that the magnitude of changes in glucose-reversible cytochalasin B binding sites on EAT cell surface was closely correlated with and sufficiently accounted for the changes in the maximal rate of glucose uptake. Michaelis constant (Km) for glucose uptake and apparent dissociation constant (Kd) for cytochalasin B binding remained relatively unchanged in all cases studied. This indicated that there was no qualitative change in the turnover and/or

affinity for glucose of the carrier of EAT cells during these drug treatments. Our findings that the suppression of glucose uptake of EAT cells by methotrexate were reversed by thymidine administration and that the glucose uptake of EAT cells could be inhibited by actinomycin D administration might suggest that the inhibition of DNA, RNA and protein synthesis might have constituted the primary chain of events leading to reduced glucose carrier production in EAT cells during the treatment of anti-tumor agents.

The binding of cytochalasin B on L929 cells, thymocytes, spleen cells and macrophages of mouse could not be displaced by high concentration of glucose in the incubation. These observations might imply that cytochalasin B might not serve as marker of glucose carrier of these cells.

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ABBREVIATIONS

AET	2-Aminoethylisothiouronium bromide
BCG	Bacillus Camette-Gurein
Bo	Density of CB binding sites
CB	Cytochalasin B
CPE	Cytopathic effect
cpm	Count per minute
C. parvum	Corynebacterium parvum
DOG	2-Deoxy-D-glucose
EAT	Ehrlich ascites tumor
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
i.p.	Intraperitoneally
i.v.	Intravenously
Kd	Apparent dissociation constant
Km	Apparent half-saturation constant (Michaelis constant)
LPS	Lipopolysaccharide
MLM	Mycobacterium lepraemurium
MTX	Methotrexate
NBTGR	S-p-Nitrobenzyl-6-thioguanosine
PBS	Phosphate buffered saline
Poly I:C	Polyriboinosinic : polyribocytidylic acid
POPOP	2,2'-p-Phenylene-bis(5-phenyloxazole)
PPO	2,5-Diphenyloxazole
SDS	Sodium dodecylsulfate

TCA	Trichloroacetic acid
TdR	Thymidine
TNF	Tumor necrosis factor
TNS	Tumor necrosis serum
Vmax	Maximal rate of uptake
UdR	Uridine

CHAPTER ONE

GLUCOSE TRANSPORT IN L929 AND EHRLICH ASCITES TUMOR CELLS

ORIGIN OF EHRLICH ASCITES TUMOR

Ehrlich ascites tumor (EAT) was derived from a spontaneous murine mammary adenocarcinoma (Ehrlich & Apolant, 1905) and was first converted from solid form into ascites form by Lowenthal and Jahn (1932) and carried in outbred mice by serial intraperitoneal passage. In our laboratory, we use the tetraploid subline Ny Klein strain which was established by Klein and Klein (1956). Ehrlich ascties tumor cells can be grown in mice and transplanted to permanent suspension culture and vice versa. For in vivo studies, 10 - 5 x 10^6 tumor cells can be transplanted intraperitoneally into the mouse and the maximal growth of tumor (about 2 x 10^9 cells) can be attained 8 - 9 days after tumor implantation. For the passages of tumor cells in culture, 8 - 10 fold dilution with fresh medium is performed every 3 - 4 days. The limit for Ehrlich ascites tumor cells grown in culture is about 10^6 cells/ml and the generation time is quite constant with average length of 22 hours before reaching the growth plateau.

ORIGIN OF L929 CELLS

NCTC clone 929 was derived by Sanford, Earle and Likely (1948) from the parental strain L established in 1940 by Earle (1943). Strain L was one of the first cell strains to be established in continuous culture, and clone 929 was the first cloned strain developed. The parent L strain was dervied from normal subcutaneous areolar and adipose tissue of a 100 day-old male C3H/An mouse, and clone 929 was established by the capillary technique for single cell isolation from the 95th subculture generation of the parent strain. Since its origin, the cells have been maintained in different media, exhibiting different properties and were considered to be derivatives or sublines. The monolayer L929 cells are cultured in RPMI 1640. The passages of cells are carried out every 4 - 5 days with the help of trypsinization to round up and detach the cells from the surface of the flask. The cells can be adjusted again to 10^5 cells/ml and seeded into 25 cm^2 culture flask. The cells will resume the spindle shape and grow to confluency after 3 - 4 days.

GLUCOSE METABOLISM IN L929 AND EHRLICH ASCITES TUMOR CELLS

It has been as early as 1956 when Warburg discovered that tumor cells usually have a characteristically distinct glucose metabolism from those of normal cells. They mainly metabolize glucose via glycolysis even in the presence of oxygen. A term called 'aerobic glycolysis' was devised to

signify this abnormal kind of glycolysis.

Ehrlich ascites tumor cells metabolize glucose to lactate by means of this kind of aerobic glycolysis and were found to have higher glucose uptake rate than normal cells (Crane, Field & Cori, 1957; Saha & Coe, 1967; Rubin, 1971; Kaminskas, 1979). In later times, Ehrlich ascites tumor cells were found to depend highly on glycolysis for generation of ATP (Lazo, 1981) and the deprivation of glucose supply brought forth a decreased adenylate energy charge (Live & kaminskas, 1975).

Little has been reported for the glucose metabolism of L929 cells. It is generally believed that L cells can grow in glucose limited medium provided they are supplied with other essential elements (see Flow Manual, 1974).

GLUCOSE UPTAKE IN L929 AND EHRLICH ASCITES TUMOR CELLS

The glucose uptake in various tumor and normal cell lines including Ehrlich ascites tumor cells is mediated through two distinguished mechanisms, namely unsaturable diffusion and saturable facilitated diffusion (Saha & Coe, 1967; Kolber & Lefevre, 1967; Lieb & Stein, 1970; Plagemann & Richy, 1974; Hatanaka, 1974). Unsaturable diffusion is the simple diffusion of glucose from extracellular environment into intracellular environment due to the gradient between these two environments. The saturable diffusion is thought to be

mediated by carrier molecules that reside on the cell membrane and follows Michaelis-Menten kinetics. It may adequately described by kinetic parameters Vmax (maximal rate of uptake) and Km (apparent half-saturation constant). For the measurement of these kinetic parameters, radioactive glucose analogs (eg. 2-deoxy-D-[^{3}H]-glucose, 3-O-methyl-D-[^{3}H]-glucose etc.) are employed in the experiments. Since the radioactivity can be determined accurately and the glucose analogs are non-metabolized, the parameters can be evaluated with higher accuracy. Competitive inhibitors, for example cytochalasin B, are used to determine the passive diffusion due to the inhibition of carrier activity by these inhibitors. The difference between the treatment with and without inhibitors can be ascribed to the carrier-mediated uptake.

In the case of L929 cells, same rationale was employed for the measurement of facilitated glucose uptake. Since the cells are monolayer type, the glucose uptake experiments were performed in wells of culture plate where L929 cells were seeded. The results are expressed in nmoles 2-deoxy-D-glucose uptake per mg protein.

CYTOCHALASIN B BINDING AND GLUCOSE CARRIERS

Cytochalasin B is a mold metabolite isolated from Helminthosporium dematioideum (Mizel & Wilson, 1972) and has been shown to prevent motility and cytokinesis in eukaryotic cells by destroying the integrity of microfilaments in the

contractile ring (Carter, 1967; Schroeder, 1968; Wessells et al., 1971). Besides the above effects, cytochalasin B was found to be a potent and reversible inhibitor of glucose uptake in animal cells (Kletzien, Perdue & Springer, 1972; Czech, Lynn and Lynn, 1973). The examples are human erythrocytes (Sogin and Hinkle, 1980), adipocytes (Karnieli et al., 1981; Suzuki and Kono, 1980), chick embryo fibroblasts (Salter & Weber, 1979) and L6 myoblast (Klip, Logan & Li, 1982). In 1974, Lin, Santi and Spudich have found two classes of binding sites for cytochalasin B in bovine red blood cells, HeLa cells and SV40 transformed mouse fibroblasts. One of these two classes of binding sites can be inhibited by D-glucose. This class of binging sites was termed 'glucose-sensitive' binding. The 'glucose-sensitive' binding site has been reported as being intimately related, if not identical to the glucose carrier in the studies made by Pinkofsky et al. (1978) and Jung and Rampal (1977). They found that in the three types of cytochalasin B binding sites in human erythrocytes, one of them, namely site I, was D-glucose sensitive. The cytochalasin B binding system of human erythrocytes have been purified (Kasahara & Hinkle, 1977; Baldwin et al., 1979) and identified as glycoproteins with apparent molecular weight of about 55,000. Reconstitution of this purified cytochalasin B binding system on liposome exhibited glucose transport activity and cytochalasin B binding in the meantime (Kasahara & Hinkle, 1977; Baldwin, Gorga and Lienhard, 1981; Lundahl et al., 1981). This

strongly indicated that cytochalasin B may be used as a molecular marker for the glucose transport protein.

CHARACTERIZATION OF GLUCOSE SENSITIVE CYTOCHALASIN B BINDING

Cuppoletti, Mayhew and Jung (1981) first characterized the glucose carrier of Ehrlich ascites tumor cells and found that cytochalasin B competitively inhibits the carrier-mediated glucose transport of Ehrlich ascites tumor cells. The inhibition constant (Ki) they found was about 5×10^{-7} M. They also found that cytochalasin E cannot inhibit the carrier-mediated glucose transport. When the cytochalasin B concentrations were up to 1×10^{-5}, the range where the inhibition develops to practical completion, three discrete cytochalasin B binding sites with different dissociation constants are distinguished (Table 1.1). These three binding sites are called L, M, and H binding sites. The cytochalasin B binding at L site shows a dissociation constant (Kd) of about 1×10^{-6} M and represents about 30% of the total cytochalasin B binding of the cell (8×10^{6} molecules/cell). The L-site binding is sensitively displaced by cytochalasin E but not by D-glucose, and is located in cytosol. The cytochalasin B binding at M site shows a Kd of $4 - 6 \times 10^{-7}$ M, represents about 60% of the total saturable binding (1×10^{7} molecules/cell), is membrane-bound and specifically displaced by D-glucose with a displacement constant of 15 mM, but not by

Table 1.1 Three Classes of Cytochalasin B Binding Sites[a]

Class	Apparent Dissociation Constant (Kd)	% of Binding	Sensitivity to Glucose	Sensitivity to Cytochalasin E
H	$2 - 6 \times 10^{-8}$ M	10	No	No
M	$1 - 6 \times 10^{-7}$ M	60	Yes	No
L	1×10^{-6} M	30	No	Yes

a Data from Cuppoletti, Mayhew and Jung (1981)

L-glucose, and is insensitive to cytochalasin E. The cytochalasin B binding at H sites shows a Kd of 2 - 6 x 10^{-8} M, represents less than 10% of the total sites (2 x 10^{6} molecules/cell), is not affected by either glucose or cytochalasin E and is non-cytosol origin. The M site was identified to be putative glucose carriers.

The binding of cytochalasin B to Ehrlich ascites tumor cells was further characterized to have the following properties (Chan et al., 1983; Chan, 1983) :

(1) High affinity for cytochalasin B. The magnitude of Kd is 10^{-7} M.

(2) Stereospecific inhibition by structurally related sugars such as D-glucose, 3-O-methyl-D-glucose, 2-deoxy-D-glucose, D-galactose and D-mannose, whereas L-glucose and D-fructose are not effective.

(3) Sensitivity to other competitive inhibitors of glucose transport such as phloretin and diethylstilbestrol.

It was found out (Chan et al., 1983) that the ability of Ehrlich ascites tumor cells to take up glucose increases progresively during the course of tumor development. Simultaneously, the density of glucose-sensitive cytochalasin B binding sites on the cell surface also increases. Since Km for uptake and Kd for binding remain unchanged throughout the course of tumor development, it appears that the changes in glucose transport by Ehrlich ascites tumor cells are

principally the result of changes in the number rather than in the affinity of transport carriers. Since there is a continuous fall of serum glucose level in tumor-bearing mice during tumor development (Pavelic et al., 1979; Chan et al., 1983), it is suggested that tumor cells compensate for the deficiency in glucose availability by increasing the number of glucose carriers (Chan et al., 1983). It was discovered that the suppression of Ehrlich tumor growth by many drugs such as methotrexate (Chan et al., 1983), N-(phosphonacetyl)-L-asparate (PALA) (Leung et al., 1984) and tumor necrosis factor (TNF) (Fung et al., 1985) paralleled with the suppression of maximal glucose uptake (Vmax) and the number of glucose-sensitive cytochalasin B binding (Bo) of the cells. This may suggest the importance of glucose transporter in Ehrlich ascites tumor cells and explain the tumor suppressing effects of these anti-tumor drugs.

Besides the majority of tumor cells, there are also some minorities of fat cells, red blood cells, tumor cell debris, immunologic macrophages and leucocytes present in the ascitic fluid. Fat cells and red blood cells have been reported to have glucose uptake ability (Kono et al., 1981; Resh, 1982; May, 1982; Pinkofsky et al., 1978). However, data concerning tumor cell debris, macrophages and leucocytes are lacking. In our experiments, we have applied steps to remove these cells from EAT cell preparation (see Chapter 3) so that their capacity of binding with cytochalasin B, if any, might not affect our estimation of glucose carrier on EAT cells.

However, it is still of interest to test whether these cells, if were not completely removed, could affect cytochalasin B binding on EAT cells. In pilot experiments, we studied the binding of cytochalasin B on these cells. The experiments on macrophages and leucocytes indicated that macrophages and leucocytes belong to a class of cells whose glucose carrier density cannot be estimated by cytochalasin B method (see Appendix I). Table 1.2 shows the results of glucose-sensitive cytochalasin B binding to dead Ehrlich ascites tumor cells. The number of functioning binding sites have been reduced to one-fifth of control value. Hence, a tentative conclusion has been drawn that dead EAT cells, macrophages and leucocytes in ascitic fluid could not affect our glucose-sensitive cytochalasin B binding on EAT cells.

Table 1.2 The glucose-sensitive cytochalasin B binding of dead Ehrlich ascites tumor cells[a]

Groups	Bo (pmoles/10^7 cells)	Kd (10^{-7} M)
EAT Cells	235.8 ± 5.87	2.20 ± 0.7
Dead EAT Cells	46.1 ± 4.65*	3.71 ± 0.6

a The detailed procedures for cytochalasin B binding are described in Chapter 3.

The results are presented as mean ± S.E.M. of triplicate determinations.

* $P < 0.001$

CHAPTER TWO

AIM OF INVESTIGATION

The tumor cells, like other cells, have many glucose carriers on their cell surface and depend highly on glucose for energy metabolism. Ehrlich ascites tumor cells have a high ability in metabolizing glucose to lactic acid by glycolysis (Warburg, 1956). It has been found out that Ehrlich ascites tumor cells contain a large amount of a kind of glucose-sensitive cytochalasin B binding sites which are closely related or even identical to the glucose carriers which are responsible for the carrier-mediated uptake of glucose (Cuppoletti, Mayhew & Jung, 1981). The content of these binding sites in Ehrlich ascites tumor cells is at least 4-fold greater than that of transformed chicken embryo fibroblasts (Salter & Weber, 1979) and that of human erythrocytes (Jung & Rampal, 1977). The high content of glucose carriers may be responsible for its tumor nature and any interference of the glucose transport system may affect the survival of tumor cells.

We have reported that Ehrlich ascites tumor cells are susceptible to many anti-tumor agents like methotrexate (MTX) (Chan *et al.*, 1983), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Leung *et al.*, personal communication), N-(phosphonacetyl)-L-aspartate (PALA) (Leung *et al.*, 1984) and

tumor necrosis factor (TNF) (Fung et al., 1985). These agents can also decrease the glucose uptake rate of the cells. To investigate whether reduction of glucose transport of tumor cells is a common mechanism of anti-tumor agents, the effect of some interferon inducers, which have been found effective against many tumor cell lines (Levy et al., 1970; Kleinschmidt, 1972), on the glucose transport system of Ehrlich ascites tumor cells were examined.

In our previous study of the effect of methotrexate and PALA on glucose carrier of Ehrlich ascites tumor cells, we have suggested that the inhibition of DNA, RNA and protein synthesis might have constituted the primary chain of events leading to reduced glucose carrier production in tumor cells (Chan et al., 1983, Leung et al., 1984). In the present thesis, we planned to examine this hypothesis. Since methotrexate can produce a "purineless" state in the tumor cells (Hryniuk, 1972), supplementation of purine or pyrimidine might reverse the anti-tumor effect of methotrexate. And besides, the supplemented purine or pyrimidine might also used by the cells as sources for DNA and RNA synthesis. If our hypothesis reveals the true situations of control of glucose carrier in Ehrlich ascites tumor cells, a reverse of suppression of glucose carrier by methotrexate should be observed when thymidine or uridine were co-administered to methotrexate-treated tumor-bearing mice. To further examine the above hypothesis, the effect of actinomycin D and cycloheximide, which are inhibitors of RNA and protein

synthesis respectively, on glucose transport of Ehrlich ascites tumor cells *in vivo* were tested.

We have investigated the effect of tumor necrosis factor on Ehrlich ascites tumor cells (Ha *et al.*, 1984a; Ha *et al.*, 1984b; Fung *et al.*, 1985). We have found that tumor necrosis serum could suppress growth of Ehrlich ascites tumor cess *in vivo* and *in vitro*. As the tumor growth was suppressed, the glucose transport was also reduced. By using cultured L929 cells as a model, we planned to study, in addition to effect on glucose transport, other effects of these nucreosis factors, namely Ca^{2+}, uridine and amino acid uptake on tumor cells.

Throughout our studies of glucose transport and glucose carrier on tumor cells, we used the conventional cytochalasin B binding method to elucidate the glucose transport mechanism of the cells (see Chapter 1). In view of a recent report by Albert (1984) which reveals that cytochalasin B does not serve as a marker of glucose transport in rabbits and erythocytes, we examined the binding of cytochalasin B to various mouse cells with the hope to investigate whether cytochalasin B is an universal ligand for glucose carrier in the cells. The mouse normal cells used were thymocytes from the thymus, spleen cells from the spleen and macrophages from the *C. parvum*-stimulated peritoneal fluid. The mouse tumor cells used was cultured L929 cells. Results of cytochalasin B

binding of these cells would be compared with those of Ehrlich ascites tumor cells.

CHAPTER THREE

MATERIALS AND METHODS

All the materials used and general methods are described here. However, some specific methods or procedures may be in the EXPERIMENTAL section of the related chapter.

MATERIALS

All the chemicals and materials listed below were of "Analytical Reagent (AR)" grade unless otherwise stated.

Chemical	Supplier
2-aminoethylisothiouronium bromide (AET)	Calbiochem.
Calcium-45 Calcium chloride in aqu. solution (16.2 uCi/mM)	Amersham
Calf serum	Gibco
Copper (II) sulphate pentahydrate	Merck
Corynebacterium parvum	Wellcome Lab.
Cytochalasin B	Sigma
[^{3}H]-Cytochalasin B (7.2 Ci/mmol)	N E N
2-Deoxy-D-glucose	Sigma
2-Deoxy-D-[^{3}H]-glucose (16.2 Ci/mmol)	Amersham
Dipyridamole	Sigma
Disodium hydrogen phosphate dihydrate	R. de Haen

Chemical	Supplier
Ethanol, absolute	Merck
Fetal calf serum	Gibco
Folin reagent	Merck
D-Glucose	Mallinckrodt
L-Glutamine	Sigma
H E P E S	Sigma
Interferon reference	Sigma
Lanthanum chloride	BDH
L-[U-^{14}C]-Leucine (342 mCi/mmol)	Amersham
L-[4,5-^{3}H]-Leucine (50 Ci/mmol)	Amersham
Lipopolysaccharide (from *E. coli*) (Serotype 0127:B8)	Sigma
Listeria monocytogenes	C. M. L.
Methanol, absolute	Ajax
L-[methyl-^{3}H]-Methionine (89 Ci/mmol)	Amersham
Methotrexate	Lederle
Mycobacterium lepraemurium	C. M. L.
S-p-Nitrobenzyl-6-thioguanosine (NBTGR)	Sigma
Penicilin (10,000 U/ml)	Gibco
Polyriboinosinic:Polyribocytidylic acid (Poly I:C)	Sigma
POPOP	Sigma
PPO	Sigma

Chemical	Supplier
Potassium chloride	Merck
Potassium dihydrogen phosphate	Merck
Potassium-sodium tartrate (general grade)	Peking
RPMI 1640	Gibco
Sodium bicarbonate	Merck
Sodium carbonate	Merck
Sodium chloride	Mallinckrodt
Sodium dodecyl sulfate (SDS)	Sigma
Sodium hydrogen phosphate	Merck
Sodium hydroxide	Mallinckrodt
Statolon (209-617B-194-3)	Lilly
Streptomycin (10,000 ug/ml)	Gibco
Thymidine	Sigma
[Methyl-^{3}H]-Thymidine (41 Ci/mmol)	Amersham
Tilorone (R10024DA)	Sigma
Toluene	Ajax
Triton X-100	Sigma
Uridine	Sigma
[5-^{3}H]-uridine (28.4 Ci/mmol)	Amersham
Zymosan	Sigma

Ajax : Ajax Chemicals, Australia.

Amersham : Amersham International Ltd., England.

BDH : British Drug House Chemical Ltd., England.

Calbiochem. : Calbiochem. (La Jolla, CA) Behring Corp., (La

Jolla, Calif).

C. M. L. : Clinical Microbiology Lab., Queen Mary Hospital, University of Hong Kong.

Lederle : Lederle Parenterals, Inc. Carolina, U.S.A.

Lilly : Lilly Laboratories, Eli Lilly & Co., Indianapolis).

Mallinckrodt : Mallinckrodt, Inc., U.S.A.

Merck : E. Merck, F.R.G.

N E N : New England Nuclear, U.S.A.

Peking : Peking Chemical Works, Peking, China

R. de Haen : Riedel. de Haen, F.R.G.

Sigma : Sigma Chemical Co., U.S.A.

Wellcome Lab. : Wellcome Research Lab., England.

METHODS

Experimental Animal

ICR strain of mice (30-35 g) were used. They were kept in air-conditioned rooms and fed standard laboratory 'chow' and tap water *ad libitum*.

Maintenance of Erlich Ascites Tumor Cell Line

A. *In vivo* maintenance of tumor cell line

Erlich ascites tumor, Ny Klein cell type, was maintained by intraperitoneal implantation in mice. Mice bearing 7-day old tumors were killed by cervical dislocation. Ascites fluid containing tumor cells were collected from peritoneal cavity by aspiration and diluted with phosphate buffered saline (PBS). The cells were collected by a table-top clinical centrifuge. The cells were then washed 5 times with half-isotonic saline to get rid of the blood cells and resuspended in PBS to 5×10^7 cells/ml. 0.2 ml cell suspension containing 10^7 cells was injected intraperitoneally into the normal mice.

B. *In vitro* culture of tumor cells

Operations : All manipulations of cell culture were performed under a laminar flow hood (Baker) with sterilized instruments and glassware.

Preparation of Culture Medium : The growth medium for all cell culture in our experiments was RPMI 1640. The medium was prepared with double-distilled water and supplemented with 25

mM HEPES and 25 mM $NaHCO_3$ according to the manufacturer's specifications. The medium was sterilized by passage through a 0.45 um Millipore filter and stored at 4°C. Sterility was tested by incubating the filtered medium in culture tubes at 37°C for 3 days. When use, the medium was supplemented with 50 units/ml penicillin, 100 ug/ml streptomycin and 10% (v/v) heated-inactivated fetal calf serum or calf serum which had been incubated at 56°C for 30 min.

Primary Culture: The culture of Ehrlich ascites tumor cells was established by collection of cells asceptically from tumor bearing mice, and immediately washed twice with sterile PBS. The washed cells were then resuspended in complete RPMI 1640 medium. Cells were seeded into 25 cm^2 culture flasks at 2 x 10^5 cells/ml.

After incubation at 37°C for 24 hours, the suspended cells were collected and seeded into new flasks. The same procedure was repeated twice and the cells were then passaged routinely.

Routine Passages of Cells : Ehrlich ascites tumor cells in culture were passaged every 3-4 days by 10 fold dilution with fresh culture medium. Cells between 16-50th passages were used for experiments.

Maintenance of L929 Cells

The growth medium used is the same as in the culture experiment of Ehrlich ascites tumor cells. 10^5 L929 cells, were seeded into 25 cm^2 culture flask and passaged routinely.

Rountine Passage of L929 Cells : L929 cells were passaged every week. The medium was removed from the culture flask and

0.25% trypsin solution was added adequately to cover the monolayer of cells. After incubation at 37°C for 1 minute, the cells became detached from the surface of the flask. The cells were resuspended with growth medium and any aggregation of cells were broken down by a pipette. The viable cells were counted by trypan blue exclusion method and cell concentration was adjusted to about 10^5 per ml and incubated at 37°C in 0.5% CO_2.

2-Deoxy-D-[^{3}H]-glucose Uptake of Ehrlich Ascites Tumor Cells

The method as described in Leung et al. (1984) was followed. Ehrlich ascites tumor cell suspension (2×10^7/ml) in PBS) were equilibrated to 37°C. At zero time, 0.2 ml cell suspension was mixed with 0.2 ml of pre-warmed 2-deoxy-D-[^{3}H]-glucose (1 uCi/umol) to give final concentrations of 0.1875 to 1.0 mM. Reaction was stopped after 6 sec by transferring 0.2 ml of mixture to 1 ml ice-cold PBS supplemented with 20 mM 2-deoxy-D-glucose. Cells were collected by centrifugation at 15,000 g for 10 sec in an Eppendorf 5414 microcentrifuge. The supernatant was removed by aspiration and the cells were washed with 1 ml of the same buffer. Cell lysis was accomplished by the addition of 0.2 ml 0.1% Triton X-100. One ml of Triton X-toluene scintillant was added and the radioactivity counted in a Beckman LS-7000 liquid scintillation counter. Non-specific diffusion was corrected by subtracting the uptake in the presence of 10 uM (final concentration) cytochalasin B. The kinetic parameters Vmax

and Km, representing respectively the maximal uptake rate and apparent half-saturation constant for the specific transport process, were determined by double-reciprocal plot.

2-Deoxy-D-[^{3}H]-glucose Uptake of L929 Cells

10^5 L929 cells, in 0.5 ml complete RPMI 1640 medium, were seeded into each well of a 24-well plastic tissue culture plate (Falcon). The monolayer of L929 cells was then washed twice with PBS (pH 7.2) and equilibrated to 37 °C. At zero time, 0.5 ml prewarmed 2-deoxy-D-[^{3}H]-glucose (1 uCi/umol) in PBS was added to give final concentrations of 0.5 - 3.0 mM. Reaction was stopped after 5 min by aspirating off the medium and rapid washing the cells three times with 1 ml ice-cold PBS supplemented with 40 mM cold 2-deoxy-D-glucose or 10 mM 2-deoxy-D-glucose supplemented with 100 mM D-glucose. The cells were lysed by addition of 0.5 ml 0.1% Triton X-100 to individual well. Radioactivity of the lysate was counted in a Beckman LS-7000 liquid scintillation counter. Protein was determined by Lowry's method (Lowry et al., 1951).

The initial rate of uptake was plotted against the concentrations of 2-deoxy-D-glucose. The extent of the diffusion-mediated uptake was estimated graphically by drawing a line through the origin and parallel to the linear portion of the total uptake curve (Renner, Plagemann & Bernlohr, 1972; Plagemann & Richey, 1974). The uptake rate due to the transport reaction was obtained by subtracting the estimated diffusion rate due to the unsaturable component from the overall rate. The corrected rates showed the Michaelis type

kinetics and the kinetic parameters Vmax and Km were determined.

Equilibrium Binding of Cytochalasin B of Ehrlich Ascites Tumor Cells

Equilibrium binding of cytochalasin B was performed according to Cuppoletti, Mayhew and Jung (1981) with minor modifications (Chan et al., 1983). For measuring total binding, 10^7 cells in 1 ml PBS were incubated with 0.04 uCi [^{3}H]-cytochalasin B and 1×10^{-7} - 5×10^{-7} M cytochalasin B for 20 min at room temperature. After the incubation, the cells were collected by centrifugation at 15,000 g for 20 min. The radioactivities of supernatant and pellet fractions were determined.

Cytochalasin B bound was calculated as % of total. To assess the glucose-reversible binding of cytochalasin B, total binding in the absence and presence of 500 mM D-glucose were measured and the difference obtained. Maximal glucose-sensitive binding (Bo) and the apparent dissociation constant (Kd) were determined by Scatchard analysis.

Measurement of Leucine, Uridine and Thymidine Incorporation in Ehrlich Ascites Tumor Cells

Ehrlich ascites tumor cells were washed three times with sterile PBS and resuspended with culture medium to 10^6 cells/ml. 100 ul cell suspension was added into each well of a 96-well micro-titer plate (Falcon) and then mixed with 100

ul [^{3}H]-leucine, [^{3}H]-uridine and [^{3}H]-thymidine (10 uCi/ml). After incubation at 37°C for 2 hours, the cells were harvested with saline through GF/C filter by cell harvester. The filter was subsequently washed with 5% trichloroacetic acid (TCA) and absolute methanol. Filters were dried in air and placed in scintillation vials containing 3 ml toluene-scintillant. The radioactivities were measured with a Beckman LS-7000 liquid scintillation counter. Background adsorption of radioactivity was corrected by addition of radioactive precursors after incubation and immediately before the harvest of cells.

Interferon Assay

The assay was based on the method of inhibition of cytopathic effect (CPE) (Armstrong, 1981). The virus-cell system employed was vesicular stomatitis virus (VSV) - L929 cells. 0.05 ml medium containing 2 x 10^4 L929 cells was added to the well of 96-well flat-bottomed plate. After 3 hr of incubation at 37 °C with 5% CO_2, 0.1 ml of appropriately diluted serum was added to the wells in duplicate. The tray was then incubated overnight at 37 °C with 5% CO_2.

Growth medium was then removed and the cell monolayer was washed three times with warm PBS (or MEM). 0.1 ml of VSV suspension containing a standard challenge dose of 6 $TCID_{50}^*$ was added to all test wells. Virus control wells received the challenge dose of VSV, whereas the cell control wells received an equal volume of maintenance medium only.

A standard interferon preparation with known NIH units/ml which consistently gave a fixed titer was included in each

assay. Assays were done in duplicate.

The tray was then re-incubated and the result was read after 24 hr of incubation at which time the cell monolayer in the virus control wells showed 100% CPE.

*: Determination of 50% Tissue Culture Infective Dose ($TCID_{50}$) of VSV was performed by first serially diluted the VSV 3 fold in maintenance medium and 0.1 ml aliquots of each dilution were added to a group of 5 wells cells. The tray was then re-incubated and the result was read 24 hr later. Wells in which 50% or more of the cell monolayer showing cytopathic effect (CPE), characterized by cell rounding, were regarded as positive. The $TCID_{50}$ was calculated by the method of Reed and Muench (1938).

Determination of Cell Count and Viability

Cell suspension of appropriate dilution was introduced onto the ruled counting area of a haemocytometer (American Opticals) under the coverslip. The number of cells was counted with a phase contrast microscope (Nikon) using the tally counter to record numbers. Cell concentration in original suspension was calculated accordingly. For the determination of cell viability, the cells were stained with 0.5% trypan blue, and the unstained (viable) and stained (dead) cells were counted. Hence, the viability is calculated from:

$$\text{Viability} = \frac{\text{No. of viable cells}}{\text{Total no. of cells}} \times 100\%$$

CHAPTER FOUR

EFFECTS OF THYMIDINE AND URIDINE RESCUE ON TUMOR GROWTH AND GLUCOSE TRANSPORT IN METHOTREXATE TREATED EAT CELLS

INTRODUCTION

ANTICANCER ACTION OF METHOTREXATE

Methotrexate is a most widely used anti-metabolite in cancer chemotherapy. It is the 4-NH_2,N-10 methyl analogue of a class of essential cofactors called folate vitamins. The folate vitamins have to be reduced to tetrahydro form before they are biologically active. The tetrahydrofolates then receive one-carbon groups from other sources to generate the folate coenzymes. The folate coenzymes can donate their one-carbon groups to the biosynthetic reactions of thymidylic acid and purines, which are essential for DNA synthesis and cell division (Stokstad and Koch 1967).

Methotrexate is one of the antifolate agents which were put for clinical use in 1948. Antifolate agents inhibit the vital functions of folate coenzymes and hence cause a cessation of the processes of DNA synthesis and cellular replication. Many actively proliferating tissue, especially the malignant cells, have a higher rate of DNA synthesis and

cellular replication and are more susceptible to the effects of antifolates.

The structures of physiological folate cofactors and methotrexate are shown in Fig. 4.1. They share common structural features: a multi-ring pteridine group linked to a para-aminobenzoic acid and then to a terminal glutamic acid residue. Most intracellular folates are converted to polyglutamate forms, containing multiple glutamate groups linked by gamma-peptide bonds. The polyglutamate forms of folic acid are preferentially retained inside the cells and are usually more efficient cofactors than the monoglutamated compounds (McGuire & Bertino, 1981). Like the physiologic folates, methotrexate is extensively metabolized intracelluarly to polyglutamate derivatives. The common structure between methotrexate and physiologic folates renders methotrexate the ability of competitive inhibition of reductase for the generation of tetrahydrofolate. Jolivet et al. (1983) stated in their review of methotrexate that the polyglutamates formation of methotrexate causes the drug to be potentially less reversible inhibitor, to have longer retention within the cells, and to have broader range of inhibition on other folate-requiring enzymes.

The current concept of the methotrexate's mechanism of action is illustrated in Fig. 4.2. Methotrexate enters cells through the active transport system used by the physiologic circulating folate N^5-methyl-FH_4 and N^5-formyl-FH_4 (leucovorin and folinic acid) and also by passive diffusion when at high

concentrations.

After entering the cells, methotrexate quickly binds to and inactivates dihydrofolate reductase. This enzyme has a crucial role in maintaining intracellular tetrahydrofolate pools by reducing dihydrofolic acid, which is produced during thymidylate synthesis. The critical result produced by inhibition of dihydrofolate reductase is depletion of intracellular pools of reduced folate. The reaction most sensitive to folate depletion is thymidylate synthesis, which requires N^{5-10} methylene-FH_4. This reaction ceases at concentrations of 0.1 nM of methotrexate (Chabner & Young, 1973). Another folate, N^{10}-formyl-FH_4, involved in both folate-dependent steps of purine synthesis, is also depleted at approximately 0.1 uM of methotrexate (Smith et al., 1981). The lack of either thymidylate or purines blocks the synthesis of DNA. Cellular proliferation in malignant tissue is greater than in normal tissue and thus methotrexate impairs malignant growth more pronouncedly.

EFFECTS OF METHOTREXATE ON TUMOR GROWTH AND GLUCOSE TRANSPORT OF EHRLICH ASCITES TUMOR CELLS

The primary anticancer action of methotrexate is the inhibition of folate reductase and the impairment of thymidylate and purine synthesis, which in turn affects the DNA synthesis and cellular proliferation. The polyglutamate derivatives of methotrexate are important for the prolonged

Fig. 4.1 The structure of tetrahydrofolate (A) and methotrexate (B) polyglutamates.

In Panel A, one-carbon groups (R) are transported on nitrogen 5 or 10 or both.

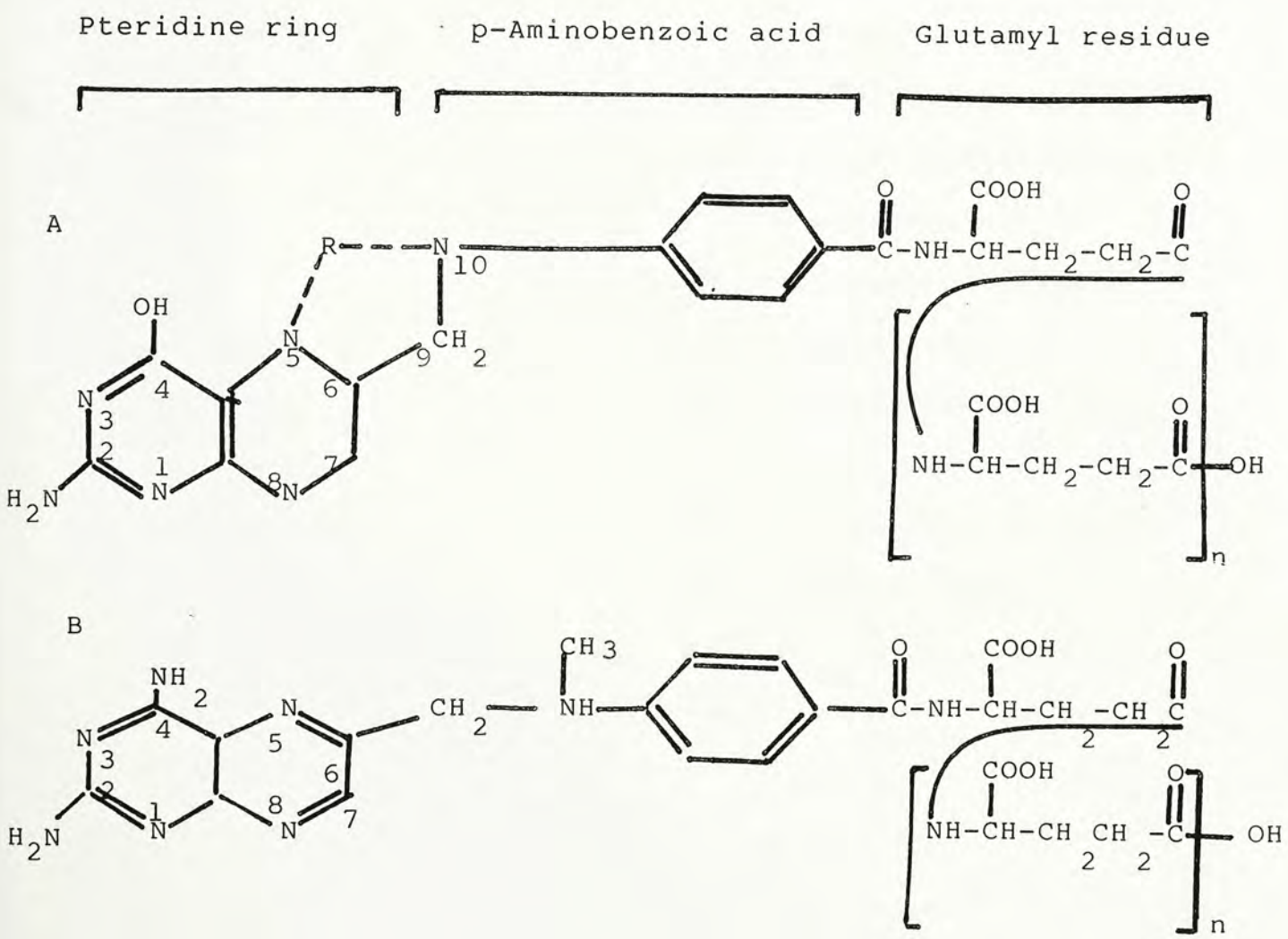
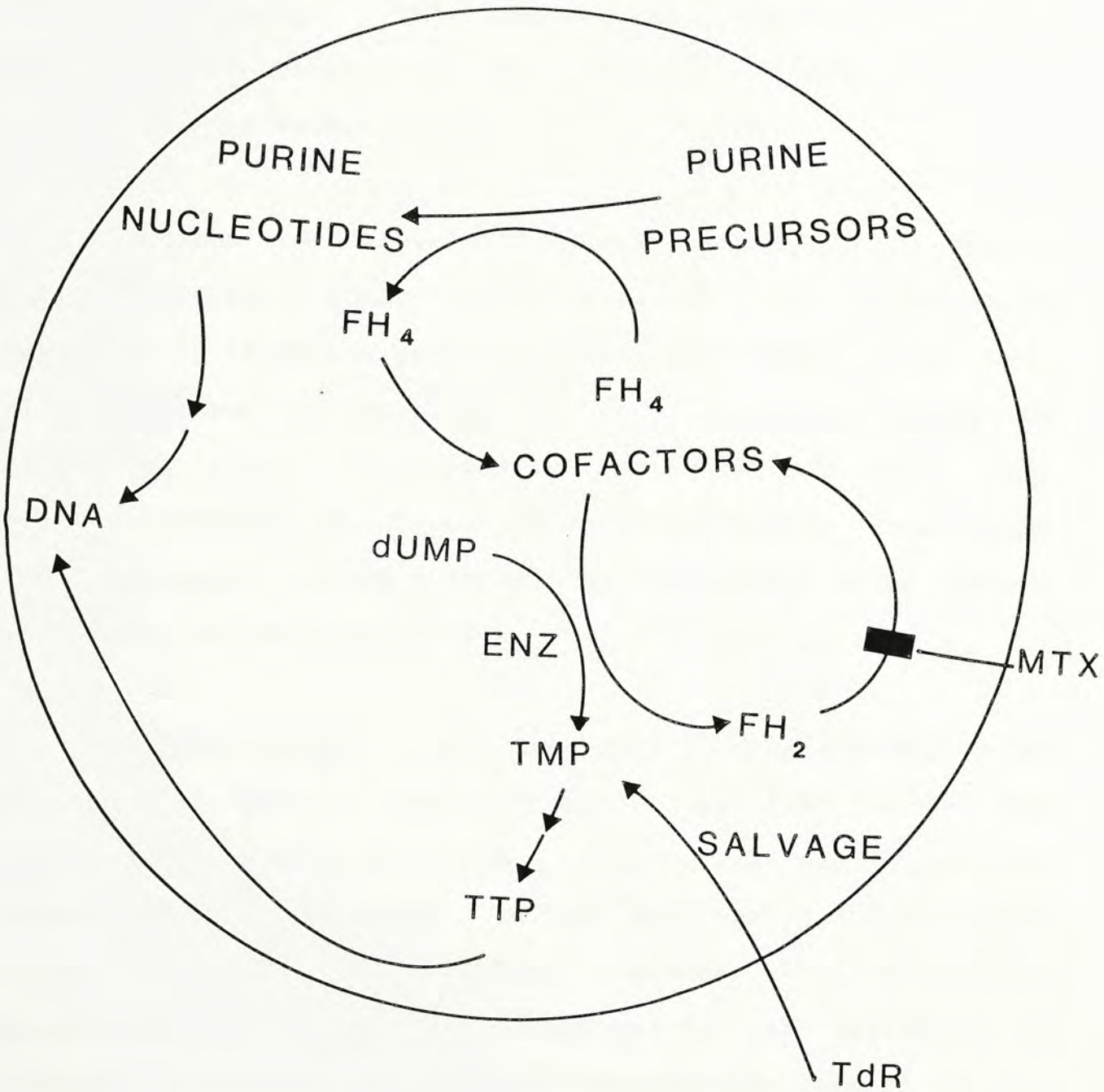


Fig. 4.2 Biochemical pathways involved in the action of methotrexate and the modulation of methotrexate toxicity by thymidine.

Abbreviations:
FH = tetrahydrofolic acid
FH = dihydrofolic acid
ENZ = thymidylate synthetase
dUMP = deoxyuridine-5'-mono-phosphate
TMP = thymidine-5'-mono-phosphate
TTP = thymidine-5'-tri-phosphate
TdR = thymidine
MTX = methotrexate

Fig. 4.2


PURINE
NUCLEOTIDES
PURINE
PRECURSORS
FH4
FH4
COFACTORS
DNA
dUMP
ENZ
MTX
FH2
TMP
SALVAGE
TTP
TdR

inhibition of dihydrofolate reductase and extended cytotoxicity (Jolivet et al., 1983). Fabres and Goldman (1984) reported that when Ehrlich ascites tumor cells were incubated with 5 uM of MTX, MTX polyglutamyl derivatives accumulated within the cells. They related the content of these polyglutamyl derivatives in the tumor cells to the cytotoxicity of methotrexate.

Kaminskas and Nussey (1978) found that glycolytic rate of Ehrlich ascites tumor cells decreased with methotrexate treatment. Adenylate pools and adenylate energy charge were also decreased in cells isolated in methotrexate containing medium in vitro. Since Ehrlich ascites tumor cells are highly dependent on glycolysis for their energy metabolism, the impairment of DNA synthesis may accompany by a severe inhibition of ATP regeneration.

We (Chan et al., 1983) have also studied the effect of MTX on the glucose transport system of EAT cells. MTX arrested EAT growth, inhibited glucose uptake, and reduced the number of glucose carrier. In both MTX-treated and untreated cells, the magnitude of changes in glucose carriers closely paralleled and sufficiently accounted for the magnitude of changes in glucose uptakes. The qualitative changes in the turnover and affinity for substrate of the glucose carrier was minimal.

THE REVERSAL OF METHOTREXATE TOXICITY BY THYMIDINE

The major action of methotrexate is the inactivation of the enzyme dihydrofolate reductase and hence the blockage of the process of dihydrofolic acid to tetrahydrofolic acid. Since intracellular dihydrofolic acid is produced only during thymidylate synthesis. The underlying rate of thymidylate synthesis is an important determinant of cytotoxicity (Moran *et al.*, 1979).

Borsa and Whitmore (1969) showed that MTX toxicity toward mouse L cells *in vitro* was enhanced by simultaneous addition of purine. This suggested that in L cells MTX led to depletion of both thymidine 5'-triphosphate (dTTP) and purines, and addition of purine resulted in a more lethal selective depletion of dTTP. In contrast with those results, Hakala (1957) reported that the inhibition of methotrexate on DNA synthesis could be prevented in cell culture by supplying the other end products of folate metabolism such as thymidine and hypoxanthine to medium which contains methionine. Hryniuk *et al.* (1969) found that MTX induced a lethal purineless state in L5178Y mouse lymphoma, and the antipurine effect was greatest in cells of rapid growth rate (1972). Tattersall *et al.* (1974) attributed the correlation between growth rate and the antipurine effect of MTX to the higher activity of thymidylate synthetase in the rapidly growing cells, which would result in faster depletion of the tetrahydrofolate cofactor pools necessary for *de novo* purine biosynthesis.

Hoglind-Semon & Grindey (1978) proposed that the mechanism of the partial protection induced by thymidine may be due to the modulation of thymidylate synthetase activity by thymidine-derived increases in the intracellular pool of dTTP. Upon metabolic conversion to dTTP, thymidine may decrease the rate of thymidylate synthetase. As the reaction catalyzed by thymidylate synthetase is the only reaction which converts reduced folates to dihydrofolate, the decrease of thymidylate synthetase activity will not deplete the reduced folate cofactors pools. Under such conditions no purine toxicity will be expressed.

The modulation of methotrexate toxicity by thymidine has been further proved by Jackson (1980) in his research with mammalian fibroblasts, lymphoblasts, and NlSl tumor cells. He stated that thymidine not only overcame the antithymidylate effect of MTX, but also antagonized the antipurine effect.

AIM OF STUDY

In pilot studies, we have observed the effect of thymidine on the reversal of cytotoxicity of MTX on EAT cells. In our previous experiments (Chan et al., 1983) on studying the effect of MTX on the glucose transport system in EAT cells, we observed that MTX reduced the number of glucose carriers on the cells. The efficacty of the drug in this respect roughly paralleled its efficacy in reducing tumor size, suggesting that MTX-induced depletion of nucleotides and the consequent inhibition of DNA, RNA and protein synthesis

might have constituted the primary chain of events leading to reduced carrier production. We planned to alleviate the anti-tumor effect of MTX on EAT cells by coadministration of thymidine *in vivo* and *in vitro* and to examine whether this treatment has any effect on the glucose transport system of EAT cells.

EXPERIMENTAL

PREPARATION OF DRUG SOLUTION

Methotrexate (5 mg vial) was reconstituted with 2 ml of sterile water and stored at -20 ℃. The solution was sterilized by passage through 0.45 um millipore filter. For *in vivo* studies, the reconstituted solution might be diluted with saline (0.85% NaCl) and used directly for injections. For *in vitro* studies, the solution was further diluted with culture medium to desired concentrations just before use.

TREATMENT OF TUMOR BEARING MICE

Mice were inoculated i.p. with 10^7 ascites tumor cells harvested from 7-day old tumors in 0.2 ml phosphate-buffered saline, pH 7.4 (PBS), on day 0. The effects of methotrexate and nucleosides were observed in groups of at least 10 mice each. In the test groups, methotrexate (1.7 mg/kg body weight) and/or thymidine/uridine (20 or 200 mg/kg body weight) were administered intraperitoneally on days 2,4 and 6 inclusively. The mice were killed by cervical dislocation on day 7. Tumor cells were washed 5 times with half-isotonic saline to remove blood cells and harvested by centrifugation. The final cell suspension was prepared in phosphate-buffered saline (PBS). Cells were counted with a haemocytometer and

resuspended in the same buffer to 2 x 10^7 cells/ml. Glucose uptake and cytochalasin B binding were then measured. For the incorporation of leucine, uridine and thymidine, the cells were resuspended in PBS to 10^6 cells/ml.

TREATMENT OF TUMOR CELLS IN CULTURE

To investigate the effect of methotrexate and/or thymidine on tumor growth in culture, 5 x 10^5 cells/ml were seeded into culture flasks. In the test groups, 0.02-20 uM methotrexate and/or 40 uM thymidine were supplemented into the culture medium. Clumps of cells were disaggregated by gentle pipetting and 0.2 ml aliquot of cell suspension was taken for cell counting. The viability was assessed by trypan blue stain.

For the study of glucose uptake, 5 x 10^5 cells/ml was seeded and incubated with 2 uM methotrexate or 40 uM thymidine in medium for 24 hours. For the reversal test, the cells were incubated with 40 uM thymidine for 2 hr before the supplementation of 2 uM methotrexate. Cells were then collected by centrifugation at 1,000 g for 5 min and washed twice with PBS. 2-deoxy-D-glucose uptake was determined as described in Chapter 3.

RESULTS

IN VITRO AND IN VIVO EFFECTS OF MTX ON GROWTH OF EAT CELLS

Fig. 4.3 shows the suppressive effect of different doses of methotrexate on Ehrlich ascites tumor size *in vivo*. Fig. 4.4 shows the suppressive effect of methotrexate (2 and 100 uM respectively) on tumor growth and viability of EAT cells *in vitro*.

EFFECT OF THYMIDINE ON TUMOR GROWTH OF METHOTREXATE TREATED EAT

Both the *in vivo* and *in vitro* reversal effects of thymidine on MTX treated EAT cells were examined. The administration of thymidine (10-100 mg/kg) can reverse the suppressive effect of methotrexate (1.7 mg/kg) on tumor growth *in vivo* (Fig. 4.5). However, the reverse is a maximum during low doses of thymidine (at the range of 20 to 40 mg/kg). At 30 mg/kg dose, thymidine could almost reverse the cytotoxic effect of methotrexate. When at higher doses, eg. 100 mg/kg, thymidine could only alleviate about 56% of the inhibition of tumor growth by methotrexate. A typical example of the thymidine effect on tumor size of methotrexate treated mice is shown in Table 4.1. Thymidine (20 mg/kg) has no effect on growth of EAT cells ($P > 0.05$). However, when thymidine was co-administered with MTX it could alleviate the suppressive effect of MTX on EAT growth.

Fig. 4.3 Dose response curve of the tumor size of EAT to different doses of methotrexate *in vivo*.
Each group consisted of 10 mice. Each mouse was inoculated with 10^7 cells on day 0. On days 2, 4 and 6, the mice received i.p. injection of indicated doses of methotrexate. On day 7, the mice were sacrified and the cells were collected for cell count. The results are presented as mean $\pm$ S.E.M. of 2 experiments.

Fig. 4.3

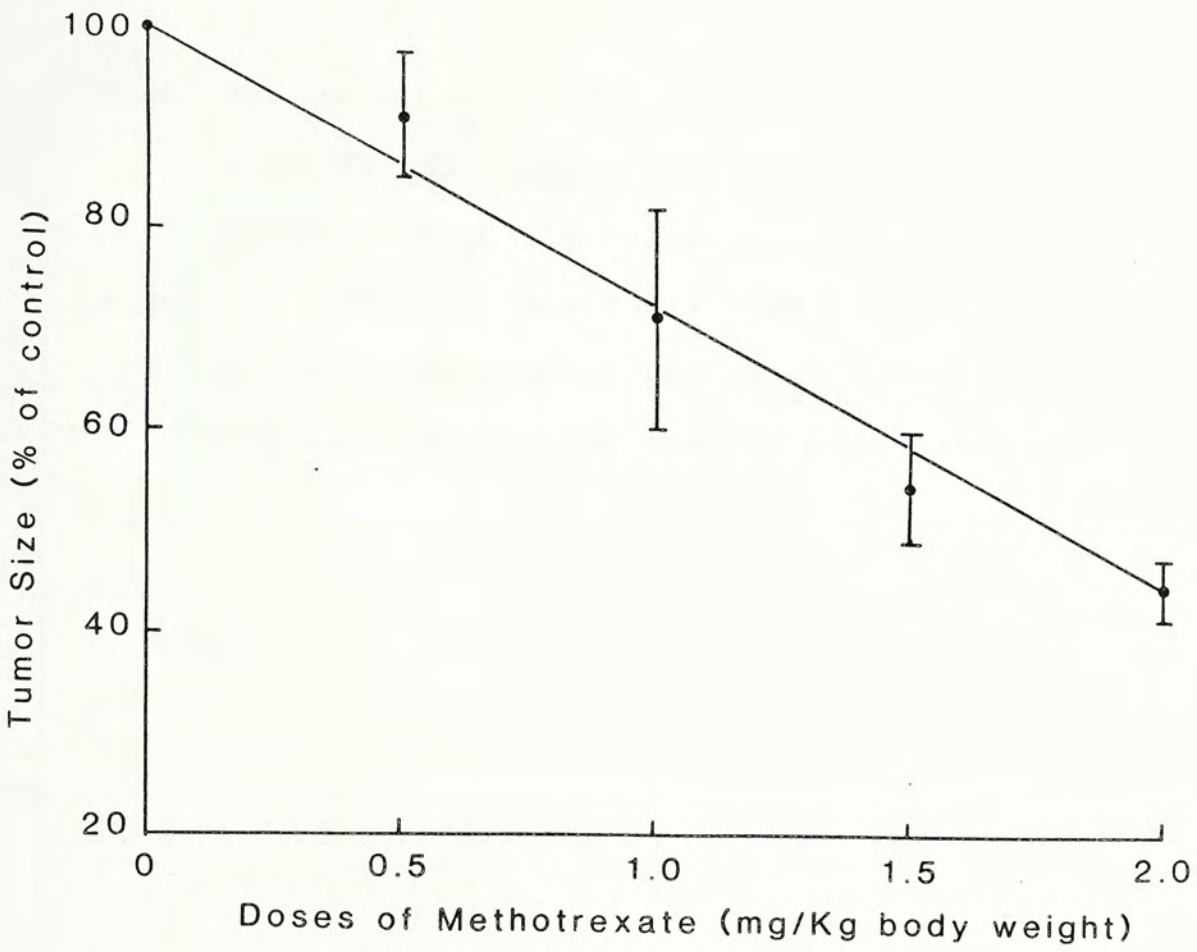

100
80
60
40
20
Tumor Size (% of control)
0
0.5
1.0
1.5
2.0
Doses of Methotrexate (mg/Kg body weight)

Fig 4.4A The effect of methotrexate doses: nil (● __ ●), 2 uM (✪ __ ✪) and 0.1 mM (▲— ▲) on total cell population of EAT cells in vitro.

4.4B The effect of the above doses of methotrexate on the viability of EAT cells in vitro.

The results are the average of 2 experiments.

Fig. 4.4A

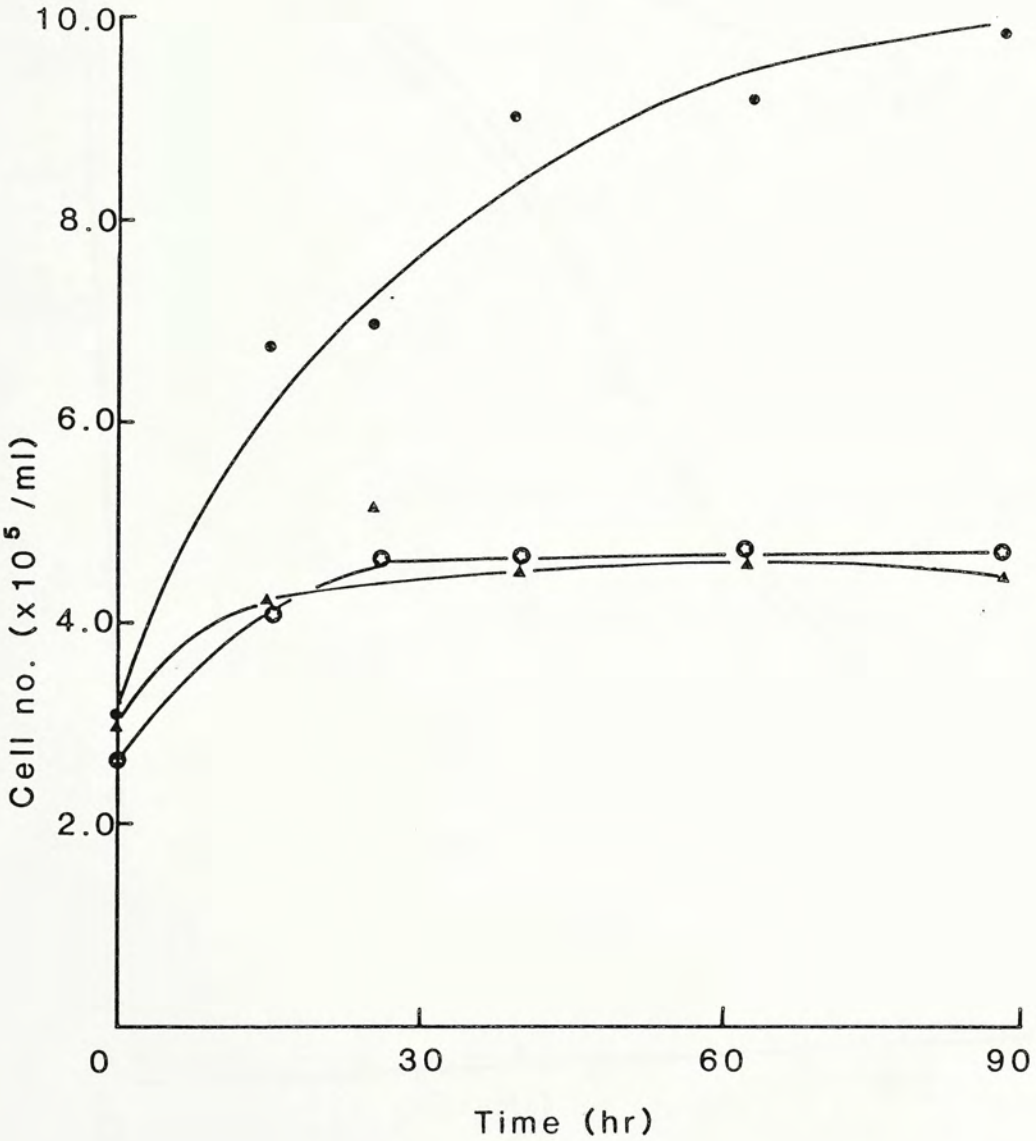


Fig. 4.4B

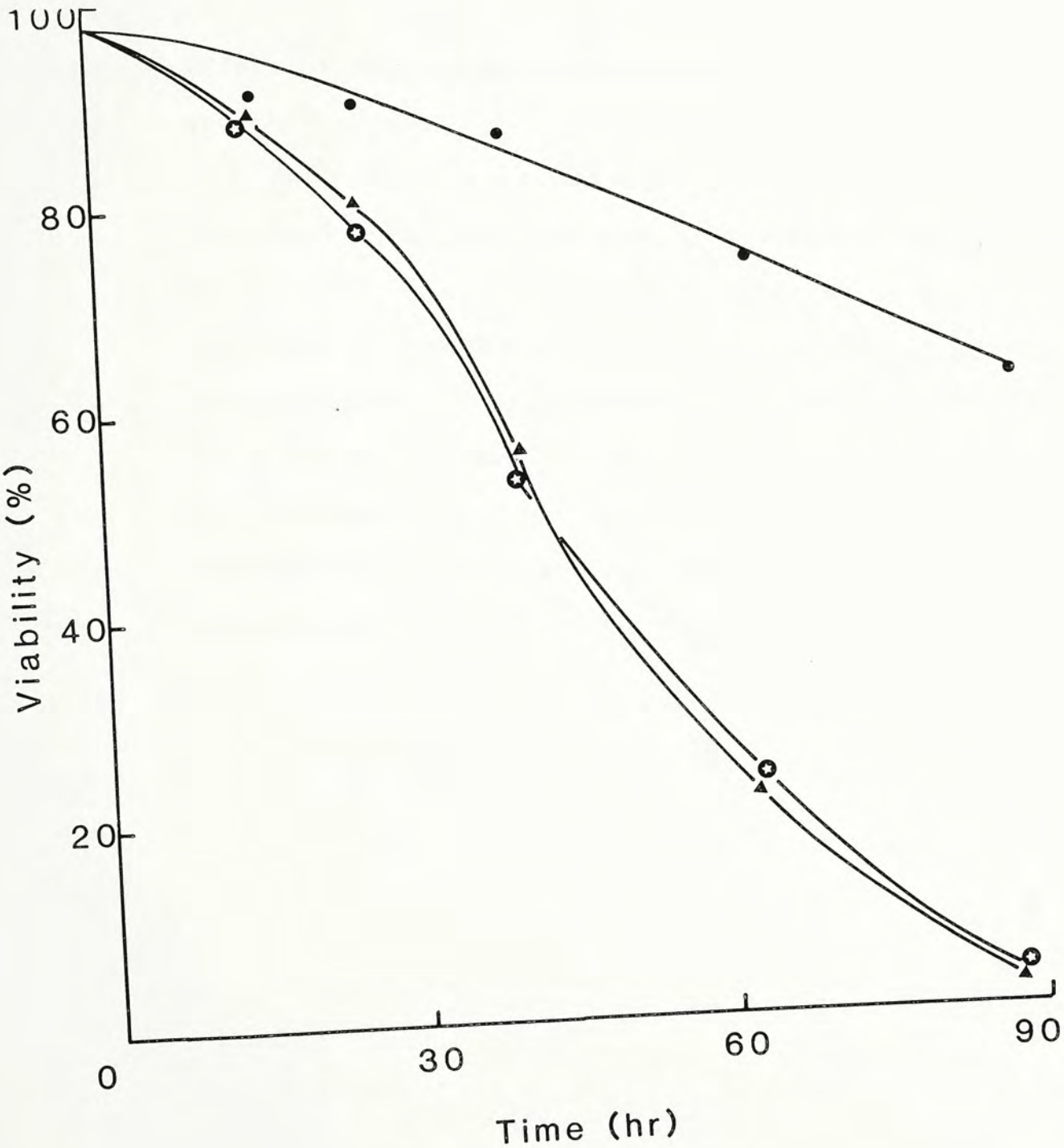


Fig. 4.5 Effect of thymidine on methotrexate treated EAT cell growth *in vivo*.

10^7 EAT cells were inoculated i.p. into the peritoneal cavities of each group (10 mice) on day 0. 0.2 ml of saline with different dosages of thymidine (10-100 mg/kg) were injected i.p. with/without 0.2 ml of methotrexate (1.7 mg/kg) on day 2, 4 and 6. On day 7, the mice were killed and the cells counted.

Results are presented as mean $\pm$ S.E.M. for 3 experiments.

Fig. 4.5

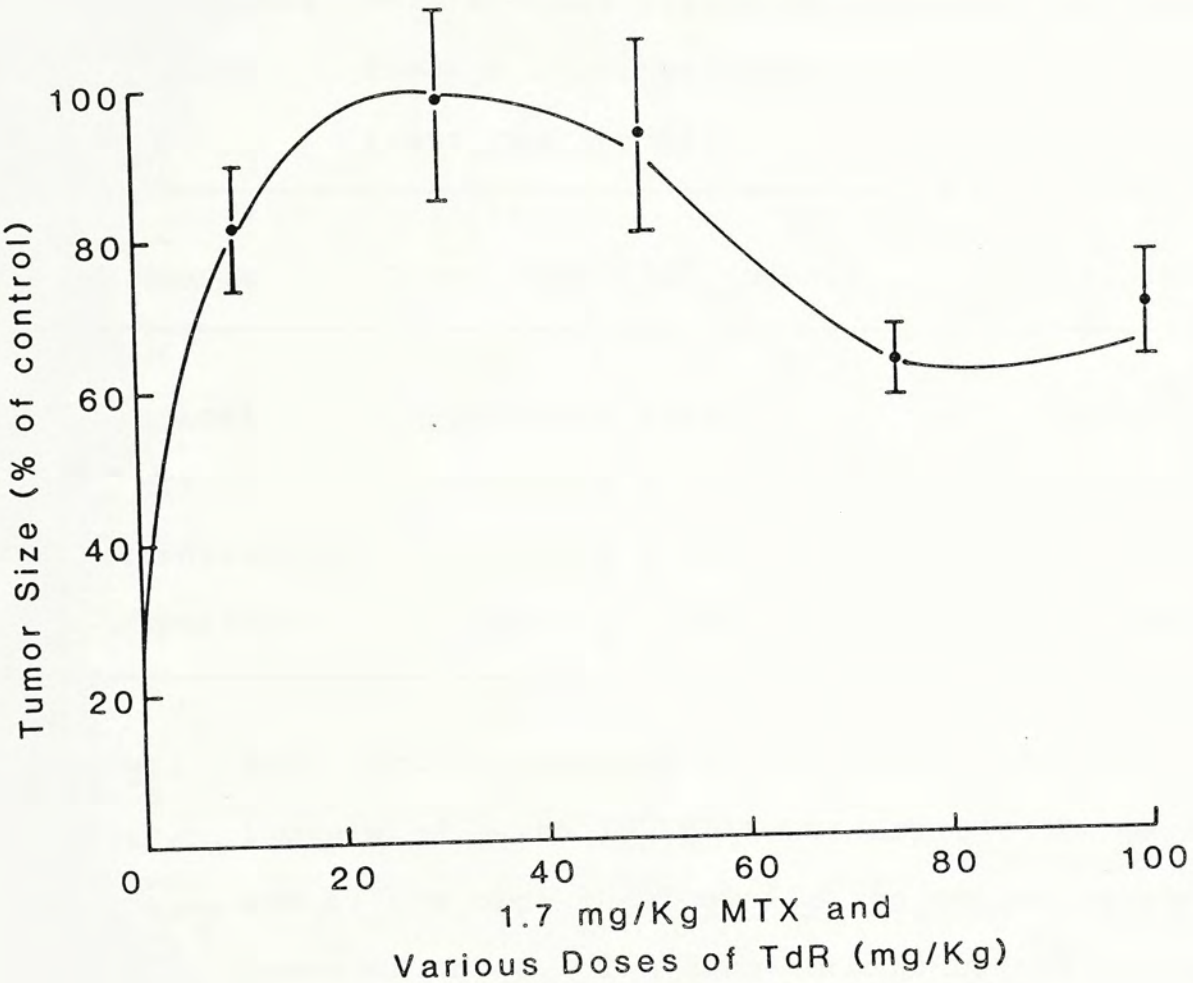

Tumor Size (% of control)
100
80
60
40
20
0
20
40
60
80
100
1.7 mg/Kg MTX and
Various Doses of TdR (mg/Kg)

Table 4.1 The reversal effect of thymidine (20 mg/kg) on tumor size of methotrexate (1.7 mg/kg) treated tumor-bearing mice.[a]

Groups	Tumor size (10^8 /mouse)	significance[b]
Control	12.36 ± 2.57	---
MTX	5.28 ± 0.61	$P < 0.001$
MTX + Thymidine	9.76 ± 3.48	$P < 0.01$
Thymidine	10.07 ± 2.73	$P > 0.05$

a Each group consisted of 10 mice. The mice were inoculated with 10^7 cells on day 0. On days 2, 4 and 6, the mice received i.p. injection of indicated doses of drugs. The mice were sacrified on day 7. The results are presented as mean ± S.E.M. for 2 experiments.

b The significance of each group was set for comparision with control, except that the value for MTX + Thymidine was set for comparison with MTX group.

Thymidine also showed positive result in *in vitro* study (Fig. 4.6). The cells population reduced with increasing doses of methotrexate. The inhibition of cell growth was significant when MTX dose reached 2 uM. It is specially interested to note that the viability of cells became significantly decreased after 50 hours incubation with MTX (Fig. 4.6 B). However, co-incubation with thymidine (40 uM) and MTX could reverse the inhibition of methotrexate on tumor growth (Fig. 4.6A) and the viability of tumor cells were also conserved (Fig. 4.6B).

EFFECT OF MTX ON GLUCOSE TRANSPORT OF EAT CELLS IN VITRO

Table 4.2 shows the effect of methotrexate (1.7 mg/kg) on glucose transport kinetics and cytochalasin B binding of cultured EAT cells. We have found that methotrexate suppressed the glucose transport and glucose carrier of EAT cells *in vivo* (Chan *et al.*, 1983). In the present experiment, we further demonstrated that MTX could significantly reduce the maximum 2-deoxy-[^{3}H]-glucose uptake rate (Vmax) ($P < 0.05$) and the number of glucose carriers (Bo) ($P < 0.001$) *in vitro*. However, this treatment did not affect the values of Km of uptake and Kd of cytochalasin B binding and these observations are also consistent with data of *in vivo* studies (Chan *et al.*, 1983).

Fig. 4.6 Effect of thymidine on tumor size and viability of methotrexate treated EAT cells *in vitro*.

A. 2×10^5 cells/ml of EAT cells were seeded in 25 cm^2 culture flasks. Total cells in the culture incubated with MTX at indicated concentrations at 26 hr (●—●), or at 50 hr (★—★), or with 40 uM thymidine and different concentrations of MTX at 26 hr (○—○), or at 50 hr (☆—☆) were assessed.

B. The viability of the treated cells were assessed by trypan blue exclusion method. Same notation as in Fig. 4.6 A.

The results are the averages of 2 experiments.

Fig. 4.6A

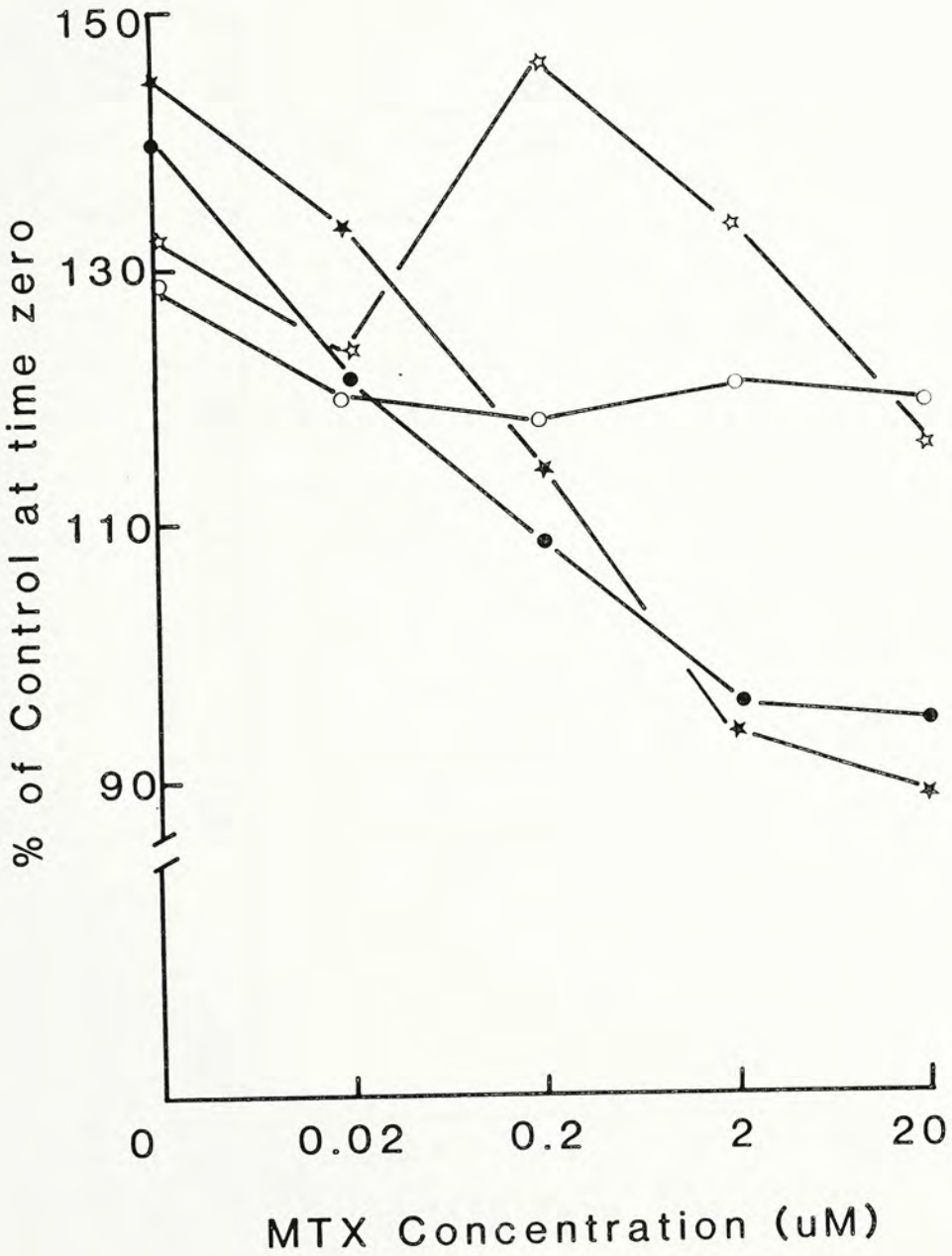

150
130
110
90
% of Control at time zero
0
0.02
0.2
2
20
MTX Concentration (uM)

Fig. 4.6B

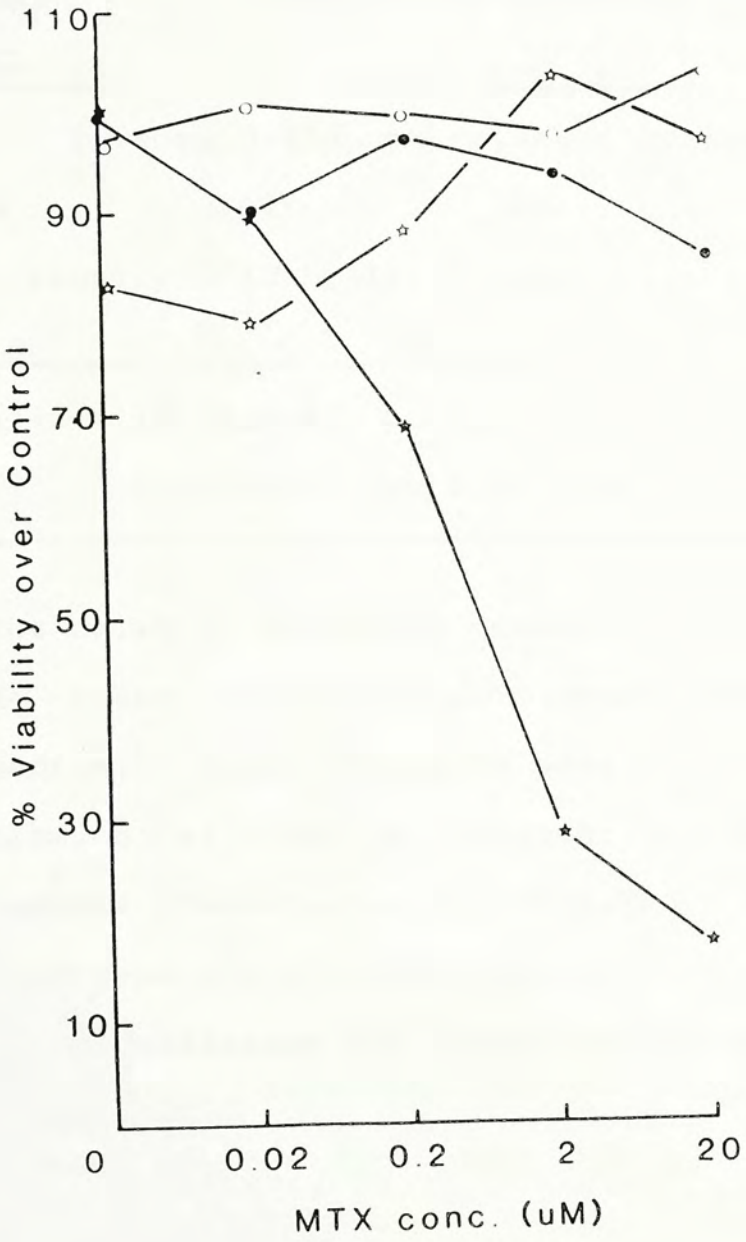

110
90
70
50
30
10
% Viability over Control
0
0.02
0.2
2
20
MTX conc. (uM)

Table 4.2 The effect of methotrexate on the glucose transport of EAT cells *in vitro*.[a]

Groups	2-deoxy-D-glucose uptake		Cytochalasin B binding	
	Vmax ($nmol/min/10^6$ cells)	Km (mM)	Bo ($pmol/10^7$ cells)	Kd (10^{-7} M)
Control	18.76±0.82	0.97±0.06	235.8±6	2.2±0.7
MTX	15.89±0.77*	0.91±0.08#	151.6±14**	2.7±0.4#

a The cells in MTX group received 2 uM MTX in medium for 24 hours. The control group received normal RPMI medium. Then the cells were collected for uptake and binding experiments as described in Chapter 3. The results presented as mean ± S.E.M. of 3 determinations in 2 separate experiments.

b The significance was determined by Student's t-test.

* $P < 0.05$

** $P < 0.001$

\# $P > 0.2$

REVERSAL EFFECT OF THYMIDINE ON GLUCOSE TRANSPORT OF EAT CELLS

The reversal effect of thymidine on glucose transport kinetics and cytochalasin B binding of EAT cells was investigated in tumor bearing mice (Table 4.3). The effect of the drugs caused a similar extent of change in both glucose uptake and cytochalasin B binding activities. While methotrexate caused about 25% reduction in both glucose uptake and cytochalasin B binding, the administration of thymidine at the same time brought the glucose transport activities back to the control levels ($P < 0.05$). Thymidine alone showed about 97% of the control levels ($P > 0.1$).

The thymidine effect was also assessed by *in vitro* study. Since the effects of drugs on the glucose uptake of EAT cells as shown in Table 4.3 paralleled to those on cytocyalasin B binding, we only examined the glucose uptake rate in *in vitro* studies. The data of 2-deoxy-D-glucose uptake (Table 4.4) showed the same pattern of the glucose uptake rate in methotrexate treated EAT cells by thymidine as in *in vivo* study.

Table 4.3 In vivo thymidine effect on glucose uptake and cytochalasin B binding of methotrexate treated EAT cells.[a]

Groups	2-deoxy-D-glucose Uptake		Cytochalasin B Binding	
	Vmax ($nmol/min/10^6$ cells)	Km (mM)	Bo ($pmol/10^7$ cells)	Kd (10^{-7} M)
Control	19.72±3.60	0.74±0.2	112.91± 7.9	1.62±0.2
MTX	14.77±0.43*	0.73±0.0	88.28± 7.3*	1.56±0.1
MTX + Thymidine	19.91±4.00**	0.84±0.4	124.73±14.3**	2.05±0.5
Thymidine	19.17±0.54***	1.28±0.1	108.33±10.1***	1.98±0.5

a Each mouse was inoculated with 10^7 cells. 0.2 ml of saline, MTX (1.7 mg/kg), MTX (1.7 mg/kg) and thymidine (20 mg/kg), thymidine (20 mg/kg) alone was injected i.p. on days 2, 4 and 6 for control, MTX, MTX + thymidine and thymidine groups respectively. Mice were sacrified on day 7. Cells were collected by exhaustive drainage. 2-deoxy-D-glucose and cytochalasin B binding were assayed as described in the text.

Values are presented as mean ± S.E.M. for triplicate

determinations.

The level of significance was evaluated by Student's t-test.

* $p < 0.01$ when compared with control

** $P < 0.01$ when compared with MTX

***$P > 0.1$ when compared with control

Table 4.4 In vitro thymidine effect on glucose uptake of methotrexate treated EAT cells [a]

Groups	Vmax (nmol/min/10^6 cells)	Km (mM)
Control	18.78 ± 0.82	0.97 ± 0.06
MTX	15.89 ± 0.77*	0.91 ± 0.08
MTX + Thymidine	20.31 ± 1.06**	1.10 ± 0.07
Thymidine	21.24 ± 3.03***	1.29 ± 0.24

a 5×10^5 EAT cells/ml were incubated with medium, 2 uM MTX alone, 40 uM thymidine followed by addition of 2 uM MTX 2 hr later, 40 uM thymidine alone for control, MTX, MTX + thymidine, and thymidine groups respectively. Cells were then washed with PBS after 24 hours and assayed for 2-deoxy-D-glucose uptake. Values are presented as mean ± S.E.M. for triplicate determinations.

The level of significance was evaluated by Student's t-test.

* $P < 0.05$ when compared with control

** $P < 0.01$ when compared with MTX

*** $p > 0.1$ when compared with control

THE EFFECT OF URIDINE ON MTX TREATED EAT CELLS

The effect of uridine on methotrexate was also studied. Experimental protocol is exactly the same as the thymidine rescue. The only change is the replacement of uridine for the thymidine in the experiment. Table 4.5 shows the results of tumor size in tumor bearing mice. Uridine either at the low dose (20 mg/kg) or at the high dose (200 mg/kg) could not exert any reverse effect on the tumor size of methotrexate treated mice. The data even shows a synergistic effect of uridine at high concentration (200 mg/kg) when administered with methotrexate ($P < 0.01$). Uridine alone, however, did not show any reduction in tumor size ($P > 0.1$).

Uridine does not show any ability to reverse the inhibition of glucose uptake and reduction of glucose carriers number by methotrexate *in vivo* (Table 4.6 and 4.7). 20 mg/kg uridine could not relieve the 20% suppression of carrier number by methotrexate (Fig. 4.7). The glucose uptake data do not show any significant reversal effect either (Fig. 4.6).

Table 4.5 The effect of uridine on tumor size of methotrexate treated tumor-bearing mice[a]

Groups	Tumor size[b] (10^8 cells/mouse)
Control	13.19 ± 3.13
MTX	4.03 ± 2.91*
MTX + Uridine (20 mg/kg)	3.51 ± 2.49+
MTX + Uridine (200 mg/kg)	1.18 ± 0.93+
Uridine (20 mg/kg)	15.62 ± 4.80#
Uridine (200 mg/kg)	15.02 ± 3.45#

a Each group consisted of 10 mice. The mice were inoculated with 10^7 cells on day 0.
0.2 ml of the drugs indicated in saline was injected i.p. on days 2, 4 and 6 post-implantation. Saline was injected for the control group. Tumor cells were harvested and counted with haemocytometer on day 7.
Results are presented as mean ± S.E.M.

b The level of significance was determined by Student's t-test.

* $P < 0.001$ when compared with control

\# $P > 0.1$ when compared with control

@ $P < 0.01$ when compared with MTX

\+ $P > 0.1$ when compared with MTX

Table 4.6 The effect of uridine on glucose uptake of EAT cells in MTX treated mice.[a]

Groups	Vmax ($nmol/min/10^6$ cells)	Km (mM)
Control	23.66 ± 0.74	1.55 ± 0.03
MTX	16.27 ± 0.08*	0.74 ± 0.001
MTX + Uridine (20 mg/kg)	17.61 ± 0.73#	0.72 ± 0.04
MTX + Uridine (200 mg/kg)	16.19 ± 0.59#	1.02 ± 0.08
Uridine (20 mg/kg)	12.60 ± 1.10*	0.62 ± 0.11
Uridine (200 mg/kg)	16.81 ± 1.16*	0.86 ± 0.10

a The experimental details are as described in Table 4.3, except that thymidine was substituted by uridine.

* $P < 0.001$ when compared with control

\# not significant when compared with MTX group

Table 4.7 The effect of uridine on cytochalasin B binding of EAT cells in methotrexate treated mice[a]

Groups	Bo (pmol/10^7 cells)	Kd (10^{-7} M)
Control	142.54 ± 8.7	2.33 ± 0.17
MTX	116.96 ± 6.3*	2.06 ± 0.19#
MTX + Uridine (20 mg/kg)	119.64 ± 6.2@	1.79 ± 0.19#
Uridine (20 mg/kg)	151.85 ±15.0#	2.36 ± 0.53#

a Experimental details were as described in Table 4.3, except that thymidine was substituted by uridine.

* $P < 0.01$ when compared with control

@ not significant when compared with MTX group

\# not significant when compared with control group

THE EFFECTS OF THYMIDINE AND URIDINE ON THE DNA, RNA AND PROTEIN SYNTHESIS OF MTX TREATED EAT CELLS

The rate of DNA, RNA and protein synthesis in various groups of treatment were estimated by radioactive thymidine, uridine and leucine incorporation respectively. Table 4.8 shows that both three precursors exhibited significantly higher incorporation rate in the MTX-treated EAT cells when the mice were injected with thymidine concurrently. In contrast, for the MTX + uridine group, when compared with MTX group, there were no changes in thymidine and leucine incorporation and even significantly lower uridine incorporation rate was observed.

Table 4.8 The effects of thymidine and uridine on precursors incorporation in MTX-treated tumor-bearing mice.[a]

Treatment	% Incorporation[b]		
	Leucine	Thymidine	Uridine
MTX + thymidine	171.59 ± 31.1*	126.60 ± 18.3**	161.43 ± 39.2*
MTX + uridine	99.11 ± 21.8#	84.33 ± 31.3#	82.04 ± 15.4*

a Experimental details are as described in Tables 4.1 and 4.5.

b Incorporation of precursors in EAT cells were performed as described in Chapter 3.

MTX group is considered as 100%.

The significance of difference was determined by Student's t-test.

* $P < 0.01$

** $P < 0.02$

\# not significant

DISCUSSION

Our results clearly demonstrate that thymidine can significantly reverse the inhibitory effect of MTX on the growth and viability of EAT cells *in vivo* (Fig. 4.5 and Table 4.1) and *in vitro* (Fig. 4.6). The mechanism of this partial protection of tumor cells induced by thymidine is not clearly understood. Hoglind-Semon and Grindey (1978) proposed that external thymidine might modulate thymidylate synthetase activity resulting in the sparing of already reduced pool of tetrahydrofolate (FH_4) in the cells. As shown in Fig. 4.2, the synthesis of deoxythymidine-5'-monophosphate (dTMP) by thymidylate synthetase involves the transfer of a one-carbon unit from a FH_4 cofactor to deoxyuridine monophosphate (dUMP) and the resultant formation of dihydrofolate (FH_2). Inhibition of dihydrofolate reductase by MTX thus interferes with the reduction of FH_2 back to FH_4, producing a loss of these FH_4 cofactors in the cell. This depletion of FH_4 cofactor would therefore occur in less frequency in cells which contained lower thymidylate synthetase activity, since this is the only reaction which converts FH_4 to FH_2. Upon metabolic conversion to deoxythymidine-5'-triphosphate (dTTP), thymidine may decrease the activity of thymidylate synthetase. This decrease may be the consequence of feedback inhibition by dTTP of the pathway of dUMP synthesis (Jackson, 1978), resulting in a decrease in intracellular dUMP pools. This hypothesis gains experimental support from the study of

Grindey et al. (1979) that 5-fluorodeoxyuridine, which is a potent inhibitor of thymidylate synthetase, can substantially reduce MTX toxicity in mice when both agents were administered by continuous infusion.

In preclinical and clinical studies, high dose of MTX can be administered safely by prolonged infusion in experimental animals (Hoglind-Semon and Grindey, 1978) and patients (Bruno, 1979) in the presence of thymidine. The molecular mechanism for this reversal of toxicity to normal cells without losing the anti-tumor activity of MTX is still not clearly defined. Our results on EAT cells clearly indicate that there are at least 20% decreases in anti-tumor activity of MTX when it is co-administered with thymidine in vivo (Table 4.1) and in vitro (Fig. 4.6A). To further evaluate the clinical applicability of this concept, i.e. rescue the normal cells by thymidine and at the same time retent the anti-tumor activity of MTX, it is important to determine the relative ratio of the dosage of MTX to that of thymidine so to provide maximum therapeutic value of MTX to cancer cells.

Our results also indicate that administration of uridine could not reverse the anti-tumor effect of MTX in vivo (Table 4.5). These results are consistent with the previous proposal (Hoglind-Semon and Grindey, 1978). External uridine has no salvage pathway to decrease the rate of thymidylate synthetase so that the intracellular FH cofactors cannot be retained by uridine administration. In contrast, excess uridine might

drive the conversion of dUMP to thymidine through the step of thymidylate synthetase and thus decrease the FH cofactors pool. In this regard, it is also of interest to determine the effect of purine on the reversal of MTX cytotoxicity on normal and tumor cells. Since inosine and hypoxanthine can enter the intacellular purine nucleotides pool through Salvage pathway, their administration should restore the FH cofactors pool in the cell, resulting in reversal of the cytoxic effect of MTX.

Results in Table 4.3 and 4.4 indicate that thymidine can also significantly reverse the inhibitory effect of MTX on glucose uptake and glucose-sensitive cytochalasin B binding in vivo and in vitro. Since the Km values of the uptake and Kd values of the cytochalasin B binding remained relatively unchanged, the alteration of the nature of the glucose carrier after MTX treatment or MTX-thymidine treatment need not be invoked. In our previous studies on the effect of MTX on the glucose transport system of EAT cells, we (Chan et al, 1983) found that the efficacy of MTX in reducing the glucose carrier number roughly paralleled its efficacy in reducing tumor size. These findings suggest that MTX-inducing depletion of nucleotides and the consequent inhibition of DNA, RNA and protein synthesis might have constituted primary chain of events leading to reduced carrier production. Our data on thymidine reversal of anti-tumor effect of MTX on EAT cells confirm the above postulation. Thymidine can protect EAT

cells to a certain degree from the cytotoxicity of MTX and at the same time increases of glucose uptake and glucose carrier density on EAT cells were oberved. Results in the precursors incorporation of MTX + thymidine group and MTX + uridine group (Table 4.8) further substantiated this view. Thymidine administration to EAT-bearing mice could reverse the cytotoxicity of MTX to tumor cells (Table 4.1). At the same time, thymidine could also increase the precursors incorporation rate of the MTX-treated cells (Table 4.8). In contrast, uridine administration could not reverse the cytotoxic effect of MTX on EAT cells (Table 4.5), and no significant increase of precursors incorporation could be observed. It is worthy to note that changes in glucose uptake and glucose carrier density was observed only in MTX-thymidine treated group (Tables 4.3 and 4.4).

Our results also indicate that uridine alone can decrease the glucose uptake to a significant level ($P < 0.001$) (Table 4.6). This is out of our expectation and may suggest that the uridine effect is a complicated one and more other factors have to be considered before we can draw a conclusive explanation. In this correction, it is of interest to note that some of purines, especially guanosine and inosine, have been demonstrated to be hyper-repressors of glucose carrier in chick-embryo fibroblasts (Gay and Amos, 1983). In this study, it has also been shown that thymidine has no effect on glucose uptake where uridine had some effect on glucose uptake by this cultured chick cells. The underlying mechanism of purines as

hyper-repressor for glucose transporter in cells is still unknown. However, it is reasonable to test the relationship between the 'purineless' condition eliciting by MTX treatment versus the glucose uptake mechanism of EAT cells. It has also been reported by Gay and Amos (1983) that the rate of glucose transport and the intracellular phosphoribosyl diphosphate (PPRibP) concentrations of chick-embryo fibroblasts are inversely correlated. We do not know whether MTX can vary the intracellular content of PPRibP and whether thymidine rescue play any role on PPRibP. The measurement of PPRibP in the cells after MTX or MTX + thymidine treatment is worth studying.

CHAPTER FIVE

EFFECTS OF INTERFERON INDUCERS ON TUMOR GROWTH AND GLUCOSE TRANSPORT IN EHRLCH ASCITES TUMOR CELLS

INTRODUCTION

Interferon inducers are any initiating entities responsible for interferon formation (Kleinschmidt, 1972). There are a variety of agents capable of inducing interferon production when given to the appropriate animals or *in vitro* cell culture system. Among these are viruses, endotoxin, bacterial extracts, double-stranded ribonucleic acids, antigens, rickettsiae, viral nucleic acid, and a heterogeneous group of synthetic compounds that range in molecular weight from the polycarboxylates (MW 30,000) to some very low molecular weight compounds like pyrimidinone interferon inducers (MW 200) (Stringefellow, 1981).

INTERFERON AND ITS ANTITUMOR ACTION

Interferons are a group of inducible glycoproteins which are produced in response to, and act to prevent replication of, an infecting virus within the invaded cells. Since the discovery of interferon by Issacs and Lindenmann (1957), a vast of researches have been focussed on this kind of cellular glycoprotein. Interferon can be produced in cells both in

tissue culture and in the intact animal. However, the classical source of interferon production are lymphoblastoid cells of the reticuloendothelial system and peripheral blood leucocytes (Interferon α), and fibroblasts (Interferon β) as well. These two kinds of interferon belong to type I interferons which are pH2 stable and generally termed viral interferons. The fact that there exist multiple molecular species (types and subtypes) of interferon with different biological functions has recently brought about the discovery of so called immune-type (type II) interferon γ . This molecular variant of interferon is in fact a lymphokine which can be produced by sensitized lymphocytes with viable Mycobacterium bovis, strain BCG or whole dead tubercle bacilli etc. and challenged with specific antigen like old tuberculin, or by exposure of nonsensitized lymphocytes to mitogens such as phytohemagglutinin or concanavalin A (Neta & Salvin, 1981). This immune type interferon is speculated to have a more potent antitumor potential than the classical interferons and have synergistic antiviral as well as antitumor effects when administered in combination with classical interferons (Fleischman, Kleyn & Baron, 1980).

Certain biological and physiological properties of interferon are briefly reported here:

(1) Interferon is species specific but lacks viral specificity.

(2) Interferon is nondialyzable,nonsedimentable at 105,000g

for 2 hr, is destroyed by proteolytic enzymes and it posseses isoelectric points pH 6.5-7.5.

(3) Interferon α and interferon β are stable at pH 2, whereas interferon γ is pH 2 labile.

Although interferon γ was originally described and characterized as a specific antiviral substance, it has also been shown to affect normal cell division and function (Gresser et al., 1970). In recent times, much of the interest has been developed in the anticellular and particularly the antitumor effects of interferon.

A vast literature has accumulated on the subject of antitumor effect of interferon (Vignaux, Stanislawski and Gresser, 1984; Roos, Leanderson & Lundgren, 1984; Billiau, 1981; Koi, Motoo, Ebina & Ishida, 1981; Trapman, 1979; Ryd et al., 1979; Takeyama et al., 1978; Levy, 1970). In his review of interferons as antitumor agents, Billiau (1981) raised at least 3 possible mechanisms by which interferon may inhibit tumor proliferation. They are:

(1) Inhibition of cell growth due to a reprogrammation of cellular metabolism. At least two intracellular enzymes with regulatory capacity for protein synthesis are induced by interferon treatment of cells. Both enzymes, an oligonucleotidyl synthetase and a protein kinase (Riviere & Hovanessian, 1984; Hovanessian et al., 1977; Roberts et al., 1976), originally synthesized in an apparently inactive form, are activated by interferon. Furthermore, it has been found

that interferon can inhibit tumor cell multiplication (Gresser & Tovey, 1978). Interferon appeared to block the entry of tumor cells into the S phase during cell division (Roos, Leanderson & Lundgren, 1984; Richard, Panniers & Clemens, 1981; Sokawa, Watanabe & Kawade, 1977).

(2) Alteration in cellular surfaces (Knight & Korant, 1977; Gresser & Tovey, 1978; Friedman, 1981) may, for example, diminish transport of nucleosides and thus impair the nucleic acid synthesis in tumor cells. Interferon treatment also results in enhanced expression of tumor-specific transplantation antigens which are held to be a target for the immune attack on tumor cells.

(3) Immunomodulation may cause stimulatory effect on the efferent arm (antibody-dependent or purely cellular effector mechanisms). Though interferon shows direct effect on tumor cell proliferation *in vitro*, the serum concentration *in vivo* seems rather low compared to the dose of interferon needed for tumor cell inhibition (Strander & Einhorn, 1977). Thus, part of the effect of interferon might be related to its potent effects on the immune system. Evidences have shown that antibody-dependent cell-mediated cytotoxicity (Herberman, Ortaldo & Bonnard, 1979; Heron *et al.*, 1979) and cytotoxicity of specifically sensitized T-cells are enhanced. Interferon has also been shown to stimulate phagocytosis as well as tumor-cell inhibitory effects of macrophages in culture (Schultz, Papamatheakis & Chirigos, 1977), enhanced natural killer activity of unsensitized peripheral blood mononuclear

cells (Gidlung et al., 1978). All these interferon-mediated host reactions may account for the findings that interferon treatment increased the survival of mice inoculated with interferon-resistant L 1210 cells (Gresser, Maury & Brouty-Boye, 1972).

THE USE OF INTERFERON INDUCERS

The attraction of interferon for use as an antiviral and antitumor agent is based on its broad spectrum of actions. In comparison with other drugs, interferon gives rise lesser toxic effect and this effect is transient and has not produced any long term detrimental changes.

Clinical trials of interferon has had the problem of variability in preparations available. In addition, accepted methods for quantitating interferon preparation have limited to biological assays which have greater variability. The problem of purity is another difficulty in evaluating the efficacy of interferon preparations used today. Whether or not completely pure material will have identical properties to those less pure preparations commonly used remains unknown. Batch to batch reproducibility is hence a problem in evaluating the efficacy of interferon preparations in research study. Another problem confronting interferon study is the sufficient quantity of interferon preparations available.

Though the recent recombinant DNA technique is promising for a higher yield of interferon production, the current quantities of interferon preparations have not been sufficient for the experiments.

Interferon is produced by leucocytes, fibroblasts and immune system in response to certain inducers. Certain such kinds of inducers, ranging from viral vaccines to synthetic compounds and naturally occurring substances, have been found to exhibit antitumor effects (Wheelock & Dingle, 1964; Kleinschmidt et al., 1964; Field et al., 1967; Levy et al., 1969; Krueger & Mayer, 1970). These inducers also produced in tumor-bearing hosts proteins which have been characterized as interferons by identical properties to the interferons produced in vitro (Lvovsky et al., 1977). Though some (Weinstein et al., 1971) refused the correlation of antitumor effects of inducers and endogenous interferon, several investigators concluded that the antitumor effects of inducers were mediated by interferon (Sarma et al., 1969; Gresser et al., 1969a; Gresser and Bourali, 1969; De Clercq, 1977; Riviere et al., 1977). These inducers can also exert antitumor action with apparent phenomena similar to interferon action. For example, polyI:C can also enhance the cell-mediated immune response and direct action on tumor cell (Rhim & Huebner, 1971; Gresser et al., 1978). Enhanced natural killer activity (Gidlung et al., 1978) and macrophage activation (Schultz et al., 1977)was observed in mice injected with interferon inducers.

Though the administration of interferon inducers may cause worries of toxicity and side effects and also the problem of hyporesponsive state development, ie. a reduced interferon response to repeated doses of inducers (for review see Pollard, 1982), inducers hold the advantages of readily available in commercial forms, experimental results comparable, antitumor effects other than interferon action and higher degree of purity can be obtained. Experiments have been devised to minimize the toxicity of inducers and to explore the existence of other inducers with minor toxic effects. The hyporeactive state can be overcome by allowing longer intervals between successive injections of the inducers or by alternating injections of different interferon inducers (De Clercq, 1981). Since there are various types of interferon with different biological and therapeutic properties, the administration of interferon inducers may sometimes be advantageous over treatment with interferon when the type of interferon used may not necessarily be the one which interacts with tumor cells.

INTRODUCTORY REMARKS ON THE INTERFERON INDUCERS EMPLOYED IN THE EXPERIMENTS

The interferon inducers we employed in the experiment are synthetic compounds which have received much attention in interferon study (Levy, 1970; Levy et al., 1970; Talal, 1971;

Rhim & Huebner, 1971; Kleinschmidt, 1972; duBuy, 1972; Schultz et al., 1977; Lvovsky et al., 1977; Gresser et al., 1978; Gidlung et al., 1978; Trapman, 1979; DeClercq, 1981; Pollard, 1982; Nagano & Takano, 1982). The nomenclature and structural features are shown in Table 5.1.

The first one is a synthetic double-stranded RNA, polyinosinic-polycytidylic acid (Poly I:C). It has been most widely used as an interferon inducer because of its relatively low toxicity and favourable inducing properties. When administered to animals or introduced into cell cultures it exhibits a broad biological effects including the induction of interferon, an interference with the growth of several experimental malignant tumors, an adjuvant-like potentiation of cellular and humoral immune response, and the provocation of antibodies specific for double-stranded RNA (Talal, 1971). Poly I:C was discovered by Hilleman and his co-workers (1967) during their search for a potent interferon inducer. The compound is made by annealing the homopolymer polyinosinic acid with the complementary polycytidylic acid. It is one of over twenty double-stranded RNAs that have been shown to induce interferon. There has been considerable experimental evidence showing that double-stranded polyribopolymers are more active than synthetic or natural single-stranded polymer, and that DNA is much less active than RNA in interferon induction (Kleinschmidt, 1972). This has led to the postulation of a specific intracellular receptor, perhaps a protein, that recognizes specific biochemical features of the

Table 5.1 The nomenclature and structural features of the interferon inducers we employed in the experiments.

Inducer	Structural feature	Reference
Polyriboinosinic: polyribocytidylic acid (Poly I:C)	double-stranded RNA	a,b
tilorone analog R10,024DA	analog of diamine 2,7-bis-(2-diethylamino-ethoxy)-fluoren-9-one	c,d,e
statalon	a fermentation product of <u>Penicillium stoloniferum</u> polyanionic nature	c,f
AET	S,2-aminoethylisothiourea hydrogen bromide a thiol with formula $NH_2CH_2CH_2-S-\overset{NH}{\overset{\|}{C}}NH_2 \cdot 2HBr$	g

Reference:

a. Levy *et al.*, 1970

b. Talal, 1971

c. Kleinschmidt, 1972

d. Meindl, Bodo & Tuppy, 1976

e. Stringfellow, 1981

f. Kleinschmidt, Cline & Murphy, 1964

g. Lvovsky *et al.*, 1977

inducer (Colby & Duesberg, 1969).

For the antitumor action of Poly I:C, a list of animal tumors were found sensitive to Poly I:C treatment (Table 5.2, from Levy et al., 1970). The mechanism of action of Poly I:C seems to be a combination of :

(a) The mediation of interferon induced in the host. Gresser et al. (1978) have found that the use of anti-interferon globulin could nullify the anti-tumor effect of Poly I:C. Although they could not rule out the possibilty that the loss of anti-tumor effect might be by a mechanism other than the neutralization of endogenous interferon. Declercq (1977) suggested that the endogenous interferon was important to render the tumor cells sensitive to the cytotoxic effects of Poly I:C.

(b) Direct chemotherapeutic effect of Poly I:C, perhaps through integration into biosynthetic processes occuring in the tumor. Studies have shown that mice bearing a reticulum cell sarcoma showed inhibition of protein and RNA synthesis by the tumor 16 hours after the administration of Poly I:C. By contrast, normal tissues in these mice showed either enhanced or minimally altered synthesis (Levy, 1970).

Table 5.2. Effect of Poly I:C on some animal tumors. Treatment, in most cases, was 150-200 ug/mouse and three times weekly by intraperitoneal route.[a]

Tumor	% Increase in median survival over control
J96132-Reticulum cell sarcoma (c.)	130[b]
J96132-Reticulum cell sarcoma (ascites)	96[b]
Carcinosarcoma Walker 256	100
Reticulum cell sarcoma RCSL	89
Ehrlich Ascites tumor	70
S91 Melanoma	55
Fibrosarcoma	52
Bl237-lymphoma (ascites)	45
Ll210 Leukemia	42
Plasma cell YPC-1	39
Bl237-lymphoma (c.)	28
MT-1 tumor (c.)	26
Reticulum cell sarcoma ovarian	20
Leukemia P388	16
Leukemia K1964	12

a Data are obtained from review by Levy et al., 1970. With the exception of the animal with J96132 with reticulum cell sarcoma, some with Ehrlich ascites tumors, and a few with Walker carcinosarcoma, all animals ultimately died.

b Data are presented in mean day of death of the animals that died. It is reported that about 30% of all the animals treated have survived, although treatment had been stopped at about day 50.

c. = subcutaneous injection

(c) The enhancement of cell mediated defense mechanisms against foreign antigens, for example, enhancement of graft-*versus*-host (GVH) reactions, suggests that Poly I:C can through the enhancement of cellular immunity and contribute to the rejection of malignant tumors by immunologic means.

The second inducer is tilorone, which belongs to a group of low molecular weight interferon inducers. Since they are small in size, they can be cleared rapidly from the animal and are not antigenic (Stringfellow, 1981). However, some of them have undesirable toxicity and researches are developed to decrease their toxicity and improve their therapeutic activity in experimental animal. Tilorone has been shown to alter the expression of delayed-type hypersensitivity responses (Kettman, 1978) and induce lymphokines production (Neta, 1981). *In vivo* tilorone can increase natural killer cell activity and exert antitumor effect (Gidlung *et al.*, 1978). The increased macrophages induced by tilorone treatment also accounts for the enhanced resistance to acute infection with Trypanosoma cruzi in mice (James *et al.*, 1981). However, tilorone are incapable to induce interferon *in vitro*. This suggests that tilorone, like endotoxin, may not cause *de novo* synthesis of interferon, but may cause release of preformed interferon (Kleinschmidt, 1972).

The third inducer is statolon, a fermentation product of *Penicillium stoloniferum* (Kleinschmidt, 1972). This material

was identified initially as an anionic polysaccharide. Closer examination by Kleinschmidt and Ellis by sucrose gradient centrifugation of statolon showed that the activity was not associated with a polysaccharide but with a virus derived from the mycelia of P. stoloniferum. The experiment using anti-interferon globulin (Gresser et al., 1978) showed that the antitumor activity of statolon was mediated by interferon induced.

The last interferon inducer we used in our laboratory is S,2-aminoethylisothiouronium hydrobromide (AET). An interferon-like substance and virus-resistant state in the mouse was found with the treatment employing AET. Since it is radioprotective, it is active orally and diffuses through membrane carriers such as central nervous system. Pharmacological studies in man indicate that AET and related compounds can be administered with relative safety (Lvovsky et al., 1977).

THE EFFECTS OF INTERFERON INDUCERS ON EHRLICH ASCITES TUMOR CELLS

The antitumor effects of interferon and interferon inducers have been studied by many investigators (Gresser, 1972; Field et al., 1967; Krueger & Mayer, 1970; Levy et al., 1969). In their treatments of Ehrlich ascites tumor-bearing mice with interferon preparation, Gresser and Bourali (1970) found that :

1) Interferon treatment of mice before inoculation of tumor cells was ineffective.
2) Subcutaneous administration of interferon increased mouse survival, but was probably less effective than i.p. injection.
3) The efficacy of treatment in increasing mouse survival was directly proportional to the number of tumor cells inoculated.
4) Phagocytosis of tumor cells by macrophages was observed on smears obtained from the peritoneal cavities of mice treated with interferon, but not from control mice.
5) Interferon-treated mice surviving inoculation of tumor cells had an enhanced resistance to reinoculation of tumor cells.

DuBuy (1972) used Poly I:C in treating Ehrlich ascites tumor carrying mice and obtained similar results. The mice with tumor cells inoculated intraperitoneally survived significantly longer when given Poly I:C treatment i.p., as compared with subcutaneous treatment. The findings suggest that the more contact of the cancer cells with Poly I:C, the more effective the treatment. Nagano and Takano (1982) used similarly Poly I:C against EAT cells and measured significant levels of interferon in mouse serum after Poly I:C treatment.

AIM OF STUDY

In our laboratory, we measured the efficacy of interferon inducers, namely, Poly I:C, statolon, tilorone and AET in treating Ehrlich ascites tumor. Since reports concerning the effect of interferon or interferon inducers on the glucose transport system of cancer cells are lacking, we also examined the effects of these interferon inducers on glucose transport process of EAT cells. Because the effect of Poly I:C on tumor cells have been examined more in literature, its action on Ehrlich ascites tumor cells will also be investigated in greater details than those of other interferon inducers in our present study.

EXPERIMENTAL

PREPARATION OF DRUG SOLUTION

Poly I:C was dissolved in distilled water at 10 mg/ml and stored in freezer. It was diluted in phosphate-buffered saline, pH 7.4 (PBS) to desired concentration before use (Nagano & Takano, 1982). Statolone, tilorone and S,2-aminoethylisothiouronium hydrobromide (AET) were dissolved or suspended in PBS and stored in freezer. Poly I:C and statolon required sonication to aid their solubilities in PBS.

TREATMENT OF TUMOR BEARING MICE

Male mice weighing 30-35 g were inoculated i.p. with 10^7 Ehrlich ascites tumor cells harvested from 7-day old tumor on day 0 in 0.2 ml PBS. All groups consisted of at least 10 mice. The drug administration was carried out on days 2, 4 and 6 unless specifically stated. The control group received only PBS. On day 7, the mice were sacrified by bleeding from the subclavian vessels or by cervical dislocation. Sera was collected by centrifugation and stored in freezer till use. Tumor cells were collected by exhaustive drainage. Pooled cells were washed 5 times with half-isotonic saline to remove blood cells and resuspended in PBS and the viability of the cells was determined by trypan blue exclusion to ensure viability higher than 95% in all cases. For glucose uptake and cytochalasin B binding experiment, the cells were

resuspended to 2 x 10^7/ml. For the interferon assay, the sera were serially diluted and added to the wells containing L929 cells of a micro-titer plate. After the overnight incubation, the cells were challenged with vesicular stomatitis virus and the results were read after 24 hours of incubation.

For the test of serum factor which may affect the tumor cells, sera obtained from Poly I:C treated mice and control mice were injected daily to new groups of tumor-bearing mice. On day 7, the mice were sacrified. The tumor cells were harvested, washed and resuspended to the concentration for the glucose uptake and cytochalasin B binding.

For the effect of Poly I:C pre-treatment on the tumor cells, 300 ug of Poly I:C was injected intravenously to the mice every 2 days starting 2 weeks before the inoculation of tumor cells. After the tumor cells inoculation, the mice received no further Poly I:C treatment. The results were used to compare with those of control group which received PBS only, and another group which received same dose of Poly I:C only on days 2, 4 and 6 after tumor cell inoculation.

RESULTS

Since different ways of interferon inducers administration may play a significant role in tumor regression, in a pilot study we examined the most effective route of administration of poly I:C and statolon because no information is available for these two agents. Table 5.3 shows that subcutaneous injection of poly I:C and statolon seem to have the least efficiency in suppressing tumor growth. Intravenous injection of poly I:C is effective, but the most effective way is by intraperitoneal injection. Subcutaneous injection of statolon gave rise the least antitumor effect whereas subcutaneous injection of poly I:C still held certain antitumor power (30%). In the following experiments, both poly I:C and statolon were injected to mice intraperitoneally.

Since Poly I:C received much of attention in interferon induction because of its lesser toxicity and greater capacity of producing interferon, its effect on EAT were examined.

Fig. 5.1 shows the effect of different doses of Poly I:C on tumor size. Poly I:C was effective in suppressing EAT growth and the effect was in a dose-dependent manner.

When the effects of different doses of Poly I:C on the glucose transport properties were examined (Table 5.4), it was found that the glucose-sensitive cytochalasin B binding (Bo)

shows a significant decrease ($P < 0.01$) when 75 to 450 ug poly I:C were injected to each mouse. However, there was only a general reduction in glucose-sensitive binding sites and it did not show any significant dose response manner. Moreover, the kd values of the binding of cytochalasin B to EAT cells remained relatively unchanged in different poly I:C dosages.

As shown in Fig. 5.1, dosage of 300 ug/mouse could exhibit maximal suppression of EAT growth, we chose this dosage for the test of poly I:C in the following experiments. An experiment on this suggested dose of poly I:C showed that the tumor size was reduced up to 40% ($P < 0.001$). Administration of poly I:C could decrease both the maximal uptake rate (Vmax) and glucose-sensitive cytochalasin B binding by nearly 20% ($P < 0.001$). However the Km and Kd values showed no significant change (Table 5.5).

Concerning interferon titer, there was no interferon in the sera of control mice while detectable interferon titer (16 ± 8 units/ml), though relatively low, was observed in the sera of poly I:C treated mice (Table 5.5).

To test that if any antitumor effects occur in the sera of poly I:C treated mice, such sera was injected to EAT bearing mice on days 2, 4, 6 and cells were counted on day 7. Table 5.6 shows that when poly I:C treated mice sera (containing 16 ± 8 units/ml of interferon titer) were injected to mice the tumor growth was significantly suppressed as

Table 5.3 The effects of different routes of administration of Poly I:C and statolon on tumor size of EAT cells.[a]

Groups	Tumor Size (% of Control)
Poly I:C i.p.	42.12 ± 14.90*
Poly I:C i.v.	47.72 ± 21.93*
Poly I:C s.c.	62.08 ± 18.23*
Statolon i.p.	57.64 ± 15.66*
Statolon s.c.	73.03 ± 18.32**

a Each group consisted of 10 mice.
300 ug/moue in 0.2 ml PBS of poly I:C and 5 mg/mouse in 0.5 ml PBS of statolon was administered to the test groups on days 2, 4 and 6 after the inoculation of 10^7 cells on day 0. The mice were sacrified on day 7 and the cells counted according to the text.
The values are presented as mean ± S.E.M. for 2 experiments.
Abbreviation: i.p. intraperitoneal
i.v. intravenous
s.c. subcutaneous

* $P < 0.001$

** $P < 0.01$

Fig. 5.1 Dose-response curve of different doses of poly I:C on EAT tumor size.

Each group consisted of 10 mice. Each mouse was inoculated with 10^7 cells on day 0. On days 2, 4 and 6, the mice received i.p. injection of indicated doses of poly I:C. The control group received PBS.

On day 7, the mice were sacrified and the cell were collected for cell count. The values are presented as mean $\pm$ S.E.M. of triplicate determinations.

* $P < 0.01$

** $P < 0.001$

Fig. 5.1

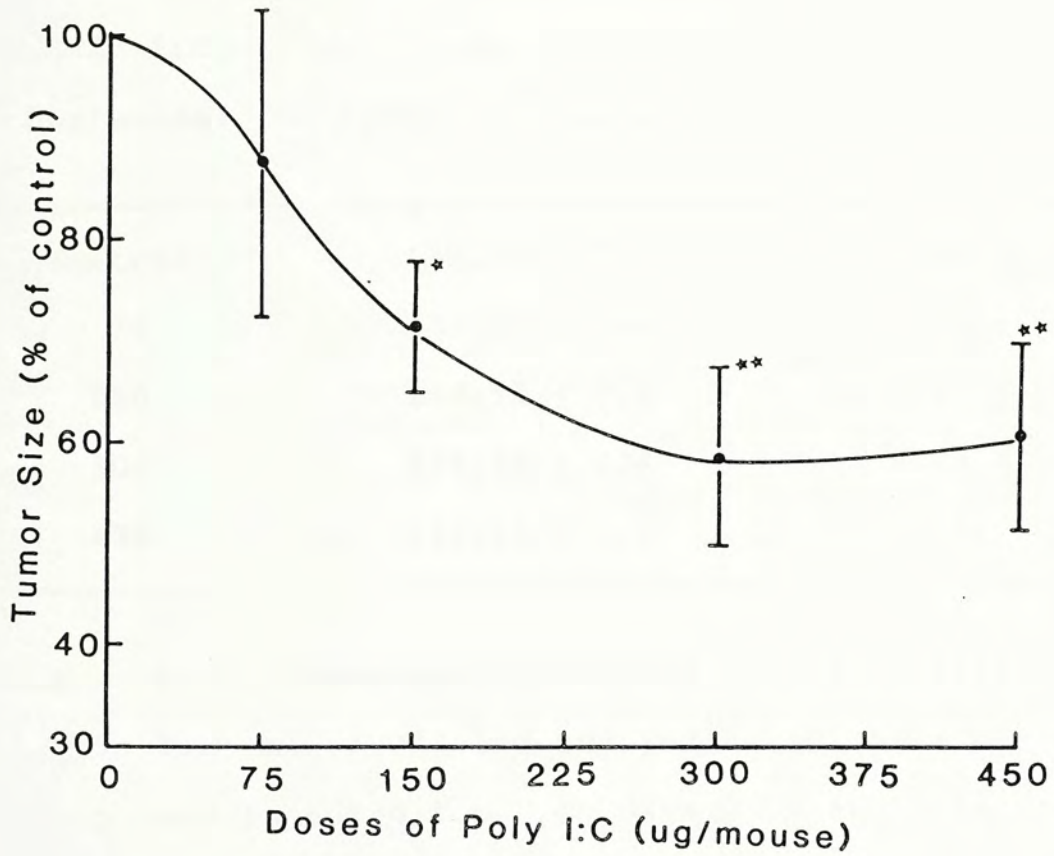

100
80
60
40
30
Tumor Size (% of control)
*
**
**
0
75
150
225
300
375
450
Doses of Poly I:C (ug/mouse)

Table 5.4 The different doses of poly I:C on glucose-sensitive cytochalasin B binding of EAT cells *in vivo*.[a]

Doses of Poly I:C (ug/mouse)	Cytochalasin B binding	
	Bo ($pmol/10^7$ cells)	Kd (10^{-7} M)
Control	175.54 ± 7.4	2.64 ± 0.2
75	133.23 ± 5.4*	1.94 ± 0.2
150	133.75 ± 7.2*	1.95 ± 0.3
300	135.76 ± 6.4*	2.13 ± 0.2
450	147.65 ± 3.2*	1.96 ± 0.2

a Each mouse was inoculated with 10^7 cells on day 0. 0.2 ml of PBS and the indicated doses of poly I:C was injected i.p. on days 2, 4 and 6 to the control and the Poly I:C groups respectively. Mice were sacrified on day 7. Cells were collected and assayed for glucose-sensitive cytochalasin B binding as described in Chapter 3.

Values are represented as mean ± S.E.M. of 3 experiments.

* $P < 0.01$ when compared with control group

Table 5.5 *In vivo* effects of Poly I:C on the tumor size and glucose transport properties of EAT cells and serum interferon titer induced.[a]

Groups	Tumor size (10^8/mouse)	Interferon Titer (reference unit/ml serum)
Control	17.95 ± 2.4	< 2
Poly I:C	10.64 ± 2.6*	16 ± 8*

	2-deoxy-D-glucose uptake		Cytochalasin B binding	
Groups	Vmax ($nmol/min/10^6$ cells)	Km (mM)	Bo ($pmol/10^7$ cells)	Kd (10^{-7} M)
Control	26.11±0.50	1.30±0.03	123.4±1.5	1.8±0.04
Poly I:C	19.39±0.49*	1.21±0.03#	101.3±3.3*	1.7±0.12#

a Each group consisted of 10 mice. The mice were inoculated with 10^7 cells on day 0. The Poly I:C group received 300 ug/mouse Poly I:C i.p. injections on days 2, 4 and 6. The control group received only PBS. The mice were bled on day 7 for interferon assay. The mice were then sacrified and cells were harvested and tumor size measured by hemacytometer. Cells were also pooled and assayed for

glucose uptake and cytochalasin B binding. The serum interferon assay and the glucose transport were determined as described in Chapter 3.

The results are presented as mean $\pm$ S.E.M. of 2 experiments. The significance of difference was determined by Student's t-test.

* $P < 0.001$

\# not significant

Table 5.6 The effect of poly I:C serum on tumor size and glucose transport activity of EAT cells and the serum interferon titer *in vivo*[a]

Groups	Tumor size (10^8 cells/mouse)	Interferon assay[b] (reference unit/ml serum)
Control Serum	13.54 ± 4.20	< 2
Poly I:C Serum	8.26 ± 3.67*	< 2

Groups	2-deoxy-D-glucose uptake		Cytochalasin B binding	
	Vmax ($nmol/min/10^6$ cells)	Km (mM)	Bo ($pmol/10^7$ cells)	Kd (10^{-7}M)
Control serum	22.77±0.3	0.79±0.1	127.28±3.0	2.48±0.2
Poly I:C serum	26.38±2.2#	0.90±0.1#	130.10±8.9#	1.86±0.3#

a Each group consisted of 10 mice. Each mouse was inoculated with 10^7 cells. 0.2 ml of control serum and Poly I:C serum was injected i.p. on days 2, 4 and 6 to control serum and Poly I:C serum

groups respectively. The mice were sacrified on day 7. The cells were collected, washed and assayed for the glucose uptake and cytochalasin B binding as described in Chapter 3.

b The original interferon titer present in the control mice serum is $<$ 2 and 16 $\pm$ 8 units/ml in the Poly I:C-treated mice serum. On day 7, serum from individual mouse was collected and assayed the interferon titer as described in Chapter 3.

Values are expressed as mean $\pm$ S.E.M. of 2 experiments.

* $P < 0.05$ when compared with control serum group

\# $P > 0.2$ when compared with control serum group

compared to that of mice receiving control sera only. However, when the interferon was assayed on day 7, titer of<2 was observed in treated group. This might reflect the relatively short half-life of interferon. When the glucose uptake and glucose carrier were examined, no significant change could be observed in the treated group as compared with control group.

The antitumor effect of Poly I:C was compared with other interferon inducers (Table 5.7). Except AET, all three inducers showed a significant antitumor effect. About 30-50% suppression of tumor growth was observed ($P < 0.001$). Of these interferon inducers, statolon seems to have greatest effect in suppressing tumor growth. The effect of Poly I:C is similar to that of tilorone. AET shows very little if any antitumor effect ($P > 0.05$) in reducing the tumor size. It should be noted that both statolon and tilorone are two interferon inducers which no interferon titer could be observed in some mice. Concerning the glucose transport activity, decreases in both uptake rate and carrier number when EAT cells were treated with statolon, tilorone and poly I:C (Table 5.8). AET has no effect in affecting the glucose transport activity and glucose carrier density ($P > 0.05$ in both cases). There is no great difference among tiorone, statolon and poly I:C under test in affecting the values of Vmax and Bo.

Pretreatment of Poly I:C (Table 5.9) gave a significant antitumor effect ($P < 0.02$) compared with control group though

the effect is less than that of the Poly I:C treatment during the course of tumor development in the host. The glucose uptake and glucose-sensitive cytochalasin B binding of both groups show a 35% decrease ($P < 0.05$) compared with control group. The Km and Kd values of both groups did not differ significantly from the control.

Table 5.7 The effects of different interferon inducers on tumor size of EAT cells and serum interferon titer in tumor-bearing mice.[a]

Groups	Tumor size (% of Control)	[b]Interferon titer (reference unit/ml serum)
Poly I:C	64.46 ± 19.56*	8 ± 4
Statolon	47.34 ± 17.11*	4 ± 4
Tilorone	70.17 ± 22.23*	4 ± 4
AET	85.43 ± 22.27#	< 2

a Each group consisted of 10 mice.
Each mice was inoculated with 10^7 cells on day 0. On days 2, 4 and 6, the mice were administered with the interferon inducers (Poly I:C , 300 ug/mouse in PBS, i.p.; statolon , 5 mg/mouse in PBS, i.p.; tilorone , 4 mg/mouse in PBS, oral; AET , 4.5 mg/mouse in PBS, i.p.). Mice were sacrified on day 7 and the cells were counted by trypan blue exclusion method.

b The interferon assay was performed as described in Chapter 3.
The results are expressed as mean ± S.E.M. for triplicate determinations.

* $P < 0.001$

\# $P > 0.05$

Table 5.8 The effects of different interferon inducers on glucose transport activity of EAT cells *in vivo*.[a]

Groups	2-deoxy-D-glucose uptake		Cytochalasin B binding	
	Vmax ($nmol/min/10^6$ cells)	Km (mM)	Bo ($pmol/10^7$ cells)	Kd (10^{-7} M)
Control	21.09±1.90	0.86±0.06	117.2±2.6	1.82±0.06
Poly I:C	19.00±0.66*	0.61±0.05	110.1±0.9*	1.85±0.02
Statolon	18.57±1.95*	0.86±0.25	102.2±5.2*	1.82±0.15
Tilorone	16.89±0.02*	0.67±0.02	105.0±2.3*	2.09±0.13
AET	17.04±3.86#	0.66±0.29	113.8±13.0#	1.82±0.38

a The procedure for drug administration and the harvest of tumor cells is the same as described in the legend in Table 5.7. The cells were collected and assayed for the glucose transport experiment as previously described. The significance of difference between test and control group was determined by Student's t-test.

* $P < 0.05$

\# not significant

Table 5.9 The effects of pretreatment with Poly I:C (i.v.), and treatment of Poly I:C (i.p.) during the development of tumor cells on EAT cells.[a]

Groups	Tumor size (10^8/mouse)
Control	14.70 ± 3.1
Poly I:C (i.p.)	8.01 ± 2.8***
Poly I:C (i.v.)	10.99 ± 2.8**

	2-deoxy-D-glucose uptake		Cytochalasin B binding	
Groups	Vmax ($nmol/min/10^6$ cells)	Km (mM)	Bo ($pmol/10^7$ cells)	Kd (10^{-7} M)
Control	38.94±8.8	1.24±8.8	190.84±2.4	2.85±0.06
Poly I:C (i.p.)	25.43±2.8*	1.13±0.3	127.31±3.4**	2.15±0.16
Pretreatment of Poly I:C (i.v.)	24.60±2.8*	0.94±0.2	125.15±6.7**	2.72±0.04

a Each group consisted of 10 mice.

The mice were inoculated with 10^7 EAT cells on day 0.

For pretreatment of Poly I:C (i.v.):

The mice were injected with 300 ug/mouse Poly I:C on alternating days started 2 weeks before the inoculation of tumor cells. Then the mice received no further injection during the development of tumor.

For Poly I:C (i.p.):

The mice were injected i.p. with Poly I:C (300 ug/mouse) on days 2, 4 and 6.

The mice were sacrified on day 7. The EAT cells were collected and ready for cell count, determinations of glucose uptake and cytochalasin B binding.

Results are presented as mean $\pm$ S.E.M. of 3 determinations.

* $P < 0.05$,

** $P < 0.02$,

*** $P < 0.001$ when compared with control group.

DISCUSSION

The results we obtained are in accordance with the results from the experiments by Gresser et al. (1978) as well as Nagano & Takano (1982). Poly I:C, statolon and tilorone were found effective against Ehrlich ascites tumor cells (Tables 5.4, 5.5, 5.7 and 5.8). AET, however, did not show any significant antitumor activity. It is difficult to say whether the antitumor effect of interferon inducers are mediated by interferon. It would appear that, not surprisingly, the inducers cause the formation of other substances in addition to interferon, which lead to suppression of tumor cells. With regard to immune system, interferon inducers, especially in the case of poly I:C, have been found to be stimulatory in anti-body production and cell-mediated immunity, while the effects of interferon seem to have been inhibitory (review see Levy and Riley, 1983). Furthermore, poly I:C, tilorone and statolon can enhance macrophage activity (James et al., 1981, Levy & Riley, 1983) and natural killer cell activity (Gidlung et al., 1978; Levy & Riley, 1983). Macrophages have been implicated in host defenses against tumor (Levy & Weelock, 1974). Natural killer cells are a subpopulation of lymphocytes that can lyse a wide variety of tumor cells. Interferon inducers should at least exert some of their interactions on tumor cells through the manipulation of immune system. However, our results indicate that interferons are an important factor for suppressing tumor growth in treated mice. For example, S,2-

aminoethylisothiouronium hydrobromide (AET) which produced very low interferon titer in mice (Table 5.7) could not suppress tumor growth. Furthermore, interferon-containing sera (with interferon titer 16 $\pm$ 8 units/ml) obtaining from poly I:C-treated mice sera (Table 5.6) could significantly suppress tumor proliferation.

In connection with the question of whether the tumor suppression brought about by the inducers is attributable solely to the interferon induced, it is important to note that the interferon titer in sera are relatively low in mice treated with poly I:C, statolon and tilorone (Table 5.7). In many cases, no interferon could be found in the sera. It has been reported that when an inducer is given to an animal, maximum levels of interferon are produced after several hours and decay of interferon is followed (Levy & Riley, 1983). Since the time we collected sera for interferon assay was 24 hrs after the last inducer injection, it is reasonable to believe that low interferon titer might be the result of its degradation. In this respect, it is also of interest to note the findings by Matsubara *et al.*, (1980) that there is a low molecular weight fraction of cell-free ascitic fluid which specifically impairs the induction of interferon in Ehrlich ascites tumor-bearing mice. Our results on pre-treatment of poly I:C on tumor-bearing mice reveal another example which indicates interferon might be the only candidate for tumor growth inhibition. Mice were pre-treated with poly I:C for 2 weeks before tumor inoculation and the growth of tumor was

monitored 1 week later. Since no inducer nor interferon might be present during tumor development, the observation that suppression of tumor occured in this pre-treatment group (Table 5.9) can only be explained by the postulation that poly I:C might produce some long-acting antitumor agent(s) or poly I:C might elicit the activation of immune system to counteract the tumor cells.

We also showed that poly I:C, statolon and tilorone could suppress glucose uptake and glucose carrier density of Ehrlich ascites tumor cells without changing the Km of uptake and Kd values of the binding (Table 5.8). It is equally difficult to know whether these decreases of glucose transport process are mediated through interferon. Since reports about the effect of interferon on glucose transport are lacking, we do not know whether interferon itself can affect glucose transport system in tumor cells. Furthermore, the possibility of indirect action of the four inducers cannot be ruled out. Treatment of poly I:C prior tumor inoculation could also suppress glucose transport of Ehrlich ascites tumor cells in a comparable degree as that of post-inocualtion treatment (Table 5.9).

Recently, Machida et al. (1984) found that blood interferon samples obtained after injection of poly I:C in mice might contain α and β interferons. In our experiments, we administered poly I:C and other inducers to mice on

alternate days (i.e. on days 2, 4 and 6 after tumor cells were inoculated on day 0). We do not know which kind of interferons might be produced *in vivo*. To investigate whether interferons have any direct effect on glucose transport system of tumor cells, it is important to obtain pure interferon-α, -β and -γ and determine their effects on tumor cells *in vitro*.

CHAPTER SIX

EFFECT OF TUMOR NECROSIS SERUM ON THE GLUCOSE TRANSPORT OF L929 CELLS

INTRODUCTION

Tumor necrosis factor (TNF) belongs to a group of macrophage products with antitumor properties (Matthews, 1983) using *in vitro* assays. These products include arginase (Currie, 1978), cytolytic factor (Adams *et al.*, 1980), tumor necrosis factor (Matthews, 1978, 1981a), human monocyte cytotoxin (Matthews, 1981b) and cytostatic factors CFI and CFII from human monocytes (Nissen-Meyer & Hammerstrom, 1982). These factors may act separately or in concert for the antitumor activities (Nissen-Meyer and Hammerstrom, 1982; Matthews, 1983).

Tumor necrosis factor was first described by Carswell *et al.* as a substance in the plasma of animals with an endotoxin shock induced by i.v. injections of *Bacillus Calmette-Guerin* (BCG) and endotoxin 2 weeks apart. Tumor necrosis factor containing plasma was found to induce necrosis of some transplantable animal tumors *in vivo* (Carswell *et al.*, 1975) and to be cytotoxic to certain cell lines but not normal cells *in vitro* (Carswell *et al.*, 1975; Matthews & Watkins, 1978).

PRODUCTION AND CHARACTERIZATION OF TNF

Tumor necrosis factor can be produced either *in vivo* or in culture. For *in vivo* production of TNF, a priming agent has to be injected into the animal. The commonly used priming agents include BCG, yeast cell wall (zymosan), *Corynebacterium parvum* (*C. parvum*) (Green *et al.*, 1977), plasmodia (Clark *et al.*, 1981), *Mycobacterium lepraemurium* (MLM) (Ha *et al.*, 1983) and *Listeria monocytogenes* (LM) (Ha *et al.*, 1985). They share a number of biological activities, such as increasing resistance to bacterial infection and to certain experimental tumors, inducing macrophage activation and proliferation, altering cellular and humoral immune reactivity, and causing heightened susceptibility to endotoxin lethality (Green *et al.*, 1977). The time of priming varies with different animals but usually is within two weeks. After priming, TNF is detected only after enough eliciting agent such as lipopolysaccharide (LPS) which is usually used, or *Pseudomonas* (Kiger, Khalil and Mathe, 1980) is given to cause death of the animal. Mouse TNF is a glycoprotein. The moeity of lipid A in LPS was found essential to TNF production while the polysaccharide portion may be involved only in delivery the lipid A moiety to TNF production cells (Ha *et al.*, 1985). The period of time for eliciting the release of TNF is between 45 min to 2 hr. The discovery that macrophage activation 2 days after *C. parvum* administration and the production of TNF when macrophage containing tissues from BCG-infected mice were

incubated with LPS for 2 hr *in vitro* suggest the source of TNF being macrophages (Green *et al.*, 1977). Moreover, pyknosis and disruption of macrophages in the greatly enlarged spleens of the mice treated with BCG followed by endotoxin and the concomitant outpouring of intracellular enzymes of lysosomal, cytosolic, and microsomal origin indicates that extensive cellular lysis occurs in the primed mouse treated with endotoxin. One example of this intracellular enzyme is NADase which is an enzyme normally bound to the membranes, is found in the serum of BCG mice after treatment with endotoxin (Green *et al.*, 1976). The lack of LPS dose-response effect in eliciting TNF implies that LPS acts in a trigger mode rather than as a primary stimulus on properly activated macrophages (Mannel, Moore & Mergenhagen, 1980).

Mouse TNF has been partially purified by Green *et al.* (1976, 1982) and Kull & Cuatrecasas (1981). Rabbit TNF is also purified to about 1000 fold by affinity chromatography (Ruff & Gifford, 1980). Recently, human recombinant tumor necrosis factor has been obtained by expression of cDNA in *Escherichia coli* (Pennica *et al.*, 1984) and the protein thus synthesized has the immunological characteristics as well as *in vitro* and *in vivo* biological properties of natural human TNF.

The effect of TNF can be assayed by necrotic response of BALB/c sarcoma Meth A *in vivo* or L929 cells *in vitro* (Carswell

et al., 1975). Recently, the cytotoxic activity of TNF has been reproducibly detected through a 1/1,000 dilution by measuring release of [^{3}H]-thymidine from prelabeled target cells (Mannel, Meltzer & Mergenhagen, 1980) and through the ability of survivor cells to incorporate the dye neutral red to measure the effect to dilutions as great as 1/64,000 with accuracy of ± 6% (Kull & Cuatrecasas, 1981).

Biochemical characterization has shown that mouse TNF protein exists in at least two forms with two different molecular weight: 15,000 (Kull & Cuatrecasas, 1981; Green et al., 1976, 1982) and a 40,000-60,000 form (Mannel et al., 1980; Kull & Cuatrecasas, 1981). They migrate electrophoretically in the α - globulin region. They are stable at pH 6-11, 56°C but inactivate at 70°C. Green et al. (1976) suggested mouse TNF was a glycoprotein because of the presence of sialic acid and galactosamine moiety. Rabbit TNF has a molecular weight of 39,000 (Matthews et al., 1980) to 67,000 (Ruff & Gifford, 1980) and have similar physicochemical properties as mouse TNF. The recently synthesized human recombinant TNF is a polypeptide related structurally to lymphotoxin. It has a relative molecular mass of 17,000 and is not a glycoprotein. Human TNF may occur naturally in multi-meric form. There are two cysteine residues in human TNF which are probably involved in a single intramolecular disulphide bond (Pennica et al., 1984).

DISTINCT TNF ACTIVITY FROM INTERFERON OR LYMPHOTOXIN ACTIVITY

Tumor necrosis serum (TNS) may contain interferon and migration inhibition factor other than TNF. Interferons, in addition to their anti-viral effects, can also exhibit anti-tumor effects (Gresser, 1977). Rabbit TNF and interferon have certain properties in common : (a) similar physicochemical characteristics, (b) production in vivo after challenge with endotoxin, (c) release from mononuclear phagocytes in vitro after short incubation periods at 37°C but not at 4°C nor on cell disruption (Matthews, 1978).

However, TNS activities appears distinct from interferon activities on the following bases (Matthews, 1979):

(a) TNF can be separated from the type I interferon of TNF serum by passage through a Cibracon Blue-agarose.

(b) Preparation of Type I interferon induced by Poly I:C or virus lacked TNF activity.

(c) Rabbit TNF has certain anti-tumor activity against human, mouse and rabbit tumor cell lines, whereas the effect of interferon is species-specific.

(d) Type I interferon has been renatured after boiling in sodium dodecylsulfate and 2-mercaptoethanol, whereas the denaturation of TNF is persistent (Kull & Cuatrecasas, 1981a).

(e) Phentolamine can effectively inhibit interferon induction but cannot antagonize the induction of tumor growth inhibition by endotoxin (Bloksma et al., 1982).

(f) The acid lability and heat stability of the TNS cytotoxin resembled the characteristics of type II interferon; however, the preparation of TNS resembled the protocol used to enrich serum for type I rather than type II interfeon (Kull & Cuatrecasas, 1981a).

In view of the heterogeneous nature of interferon, it is possible that the TNF and interferon activities share some structure-function relationship. Further comparison must await structural elucidation of both substances.

Both the TNF and lymphotoxin share certain mechanistic similarities. Target cell killing by lymphotoxin is species independent, requires prolonged exposures, and is enhanced by inhibitors of protein synthesis. Stimulation of RNA synthesis is reported for both TNS (Ostrove & Gifford, 1979) and lymphotoxin (Kunitomi et al., 1975). However, physicochemical characteristics distinguish TNS from lymphotoxin and other cytostatic lymphokines : stable at 56°C, inactivated at 70°C; pH lability to both acid and base, with a pH maximum of 8.0 (Kull & Cuatrecasas, 1981a); cytotoxicity is not inhibited by the treatment with protease inhibitors. Inhibitors of energy metabolism enhance sensitivity to lymphotoxin whereas they do not enhance sensitivity to TNS (Kull & Cuatrecasas, 1981b). TNF and lymphotoxin are now known to be antigenically distinct molecules. TNF activity on L929 cells is not inhibited by neutralizing antibodies specific for lymphotoxin (Gray et al., 1984). However, the general similarities in the mechanisms of

lymphotoxin and TNS raise the possibility that these activities may constitute a common antiproliferative leukocyte capability.

MODE OF TUMOR NECROSIS FACTOR ACTION

Many studies on the action of TNF were performed with crude serum (for review see Ruff and Gifford, 1981a). Hence, they are subject to certain reservations that the effects of TNF may have some contribution from contaminants present in TNF-containing serum.

The study of L-M cells derived from the L929 cells (Darzynkiewicz et al., 1984) has shown that the immediate effect of TNF is cytostasis, manifested as cell arrest in G2 phase of cell cycle. Shortly thereafter, the cytolytic effect become apparent; extensive cell lysis can be detected after 7 hr of exposure to TNF. After 24 hr nearly all cells are lysed. Most cells undergo lysis specifically at late stages of mitosis (telophase) or soon after cytokinesis. Nonlysed cells from cultures treated with TNF do not show any changesin nuclear chromatin, suggesting that neither DNA nor nuclear proteins are the primary targets of TNF, and metabolic changes which occur during mitosis (cytokinesis) may be responsible for increased cell sensitivity towards the cytolytic effect of TNF. Cytoskeletal disrupting agents (colchicine, Colcomid, and cytochalasin B)and inhibitors of lysosome activity (chloroquine, methylamine) are found to depress sensitivity.

The results suggest that TNF entity may need to be internalized and that lysomal activity may be necessary for antitumor effect of TNF. Similar results were obtained by Matthews and Watkins (1978). The use of antimetabolites such as sodium azide and dinitrophenol partially protects L cells from TNF (Matthews & Watkins, 1978) and the failure of rabbit TNF to kill L cells at 21°C suggest that the actively metabolizing cells have higher sensitivity to TNF or there is a possible enzymatic role of TNF. However, exact antitumor mechanisms of TNF is stil unknown and await more investigation.

Fung et al. (1985) have shown that Ehrlich ascites tumor (EAT) cells were highly susceptible to the TNS prepared by Corynebacterium parvum priming mice and elicited by LPS of E. Coli. The cytotoxic effect of TNS on EAT cells, as shown by [^{3}H]-thymidine release assay, started as early as 4 hr after incubation with TNS. TNS also inhibited EAT growth in mice in vivo and inhibit the glucose transport of EAT cells both in vivo and in vitro.

AIM OF STUDY

Since we have previously found out that mouse TNF can suppress glucose transport activity in Ehrlich ascites tumor cells, we continued the experiment on the study if TNF exerted the same effect on glucose transport activity of L929 cells as

in the EAT cells. A report by Matthews (1983) indicates that anti-tumor cytotoxin produced by human monocytes suppresses the respiration rate and increases the glycolytic and glucose uptake activities of L929 cells. Hence, it is interesting to investigate if the mouse TNF can exert similar effect on glucose transport activities of L929 cells. The study by Kull and Cuatrecasas (1981b) shows that sensitivity of L-M cells to TNF increases with elevated temperature and the presence of inhibitors (cycloheximide, actinomycin D) of RNA and protein synthesis. Ostrove and Gifford (1979) suggested that it might be due to hampered repair mechanisms of the L cells or possibly to a decrease in the synthesis of a substance which competed with TNS. Kull and Cuatrecasas (1981a) also suggested that the protein synthesis inhibitors (actinomycin D or cycloheximide) might weaken the targets by some sublethal injury, which was not directly exploited by TNS but acted synergistically to enhance toxicity. We investigated the effect of cycloheximide on TNS efficacy against L929 cells in our experiment. Also included in the experiment was the comparison of effects of TNS produced by different priming agents, namely, BCG; zymosan; Corynebacterium parvum; Listeria monocytogenes; and Mycobacterium lepraemurium on the glucose transport activity.

EXPERIMENTAL

PRODUCTION OF TUMOR NECROSIS SERUM (TNS)

Mice were injected intraperitoneally (i.p.) with 0.2 ml saline containing 1 mg *Corynebacterium parvum* or zymosan. The mice were challenged with 25 ug lipopolysaccharide W. (LPS) from *E. coli*. in 0.2 ml saline intravenously (i.v.) 7 days later. Mice were bled from subclavian vessels 90 min after LPS injection. *Listeria*-TNS was similarly obtained from mice injected i.v. with 10^4 *Listeria* in 0.2 ml saline. *Mycobacterium lepraemurium* (MLM)-TNS was obtained from mice injected i.p. with 5×10^8 bacteria 2 months before LPS challenges (Ha *et al.*, 1983). Control sera were obtained from *Listeria*-, *C. parvum*-, or LPS-injected mice. Pooled sera were incubated at 56 °C for 30 min to inactivate complement components and sterilized by filtration through 0.45 um sterile millipore filter and stored at -20 °C until use.

TNS ON VIABILITY AND MORPHOLOGY OF L929 CELLS

10^5 L929 cells were seeded in 24-well culture plate (Falcon, New York) and incubated in the presence of 1.25% (v/v) control serum, 2.5 ug/ml cycloheximide, 1.25% (v/v) TNS, or both 1.25% (v/v) TNS and 2.5 ug/ml cycloheximide respectively for 24 hr. The medium then was aspirated and 0.1 ml trypan blue was added. The viability and morphology of the

cells were studied under microsocpe.

[^{3}H]-THYMIDINE RELEASE ASSAY

L929 cells were grown in culture medium for 24 hr in the presence of 25 uCi/ml [^{3}H]-thymidine (41 Ci/mmol, Amersham). Cells were washed three times with sterile PBS and resuspended in growth medium at a concentration of 5 x 10^5 cells/ml. 0.1 ml cell suspension was added to each well of 96 well titre-plate containing 0.1ml 2% control serum, 2.5 ug/ml cycloheximide, 2% TNS, or 2% TNS with 2.5 ug/ml cycloheximide respectively at desired concentration. The mixture was incubated at 37 C for indicated time intervals and then 100 ul of supernatant was taken and added to 0.7 ml of Triton X-100-toluene scintillant. 100% releasable radioactivity (total cpm) was measured by incubating labelled cells in 0.5% sodium dodecylsulfate (SDS) for 30 min. Radioactivity was counted in a Beckman LS 7000 liquid scintillation counter. Percentage [^{3}H]-thymidine released was calculated as:

$$\% \text{ H-Thymidine release} = \frac{\text{Experimental cpm - Control cpm}}{\text{Total cpm - Control cpm}} \times 100$$

TNS ON 2-DEOXY-D-[^{3}H]-GLUCOSE UPTAKE OF L929 CELLS

The 2-deoxy-D-glucose uptake of L929 cells were determined as described in Chapter 3.

EFFECT OF TNS ON PROTEIN CONTENT OF L929 CELLS

The L-cells, at 10^5 cells/ml, were incubated in the wells of a 24-well plastic tissue culture plate (Falcon) with 0.5 ml medium alone, or medium supplemented with 2% (v/v) C. parvum-TNS, or with 2% (v/v) LPS control serum for 24 hours. The cells then were trysinized with 0.1 ml 0.25% trypsin for 5 min at 37 °C. 0.4 ml medium was added and the cells were dispersed. Then the cells were transferred to culture tubes and spinned down by a table top clinical centrifuge. The collected cells were washed once with saline and resuspended with saline into 0.5 ml. 0.2 ml of the suspension was used for cell count and 0.2 ml for protein determination by Lowry method.

RESULTS

EFFECT OF TNS ON VIABILITY AND MORPHOLOGY OF L929 CELLS

The effect of TNS on the growth of L929 cells was examined. Incubation of cultured L929 cells with 1.25% (v/v) C. parvum-TNS for 24 hours increased the mortality of the cells. Dead cells appeared as dark spots (DS) with trypan blue stain in Fig. 6.1B. For those cells which were still alive, there appeared some dark granules within the cells. The incubation of L cells with TNS in the presence of 2.5 ug/ml cycloheximide even hampered the viability of the cells (Fig. 6.1C). The cells have lost their integrity and rounded up to death. On the other hand, the incubation of cells with cycloheximide alone (Fig. 6.1D) had no significant effect on the cells as compared with control (Fig. 6.1A).

CYTOLYTIC EFFECT OF TNS

The cytotoxic effect of TNS on L929 cells was monitored by [^{3}H]-thymidine release assay to ensure the integrity of cell membrane for glucose uptake measurement. The results in Fig. 6.2 indicate that [^{3}H]-thymidine release was detectable after 24 hours of incubation. There was a progressive increase in the amount of radioactivity released into the medium from 24 to 50 hours. Hence, in the following experiments, the cells were usually incubated with TNS for 24

Fig. 6.1 The effect of C. parvum-TNS on the viability and morphology of L929 cells.

Cells were cultured in RPMI complete medium supplemented with A) Control Serum 1.25% (v/v)

B) C. parvum-TNS 1.25% (v/v);

C) TNS, 1.25% and 2.5 ug/ml cycloheximide;

D) Cycloheximide, 2.5 ug/ml

for 24 hours. Medium was then aspirated and 0.1 ml trypan blue was added. Dead cells appeared as dark spots (DS) in the figure.

Fig. 6.1A

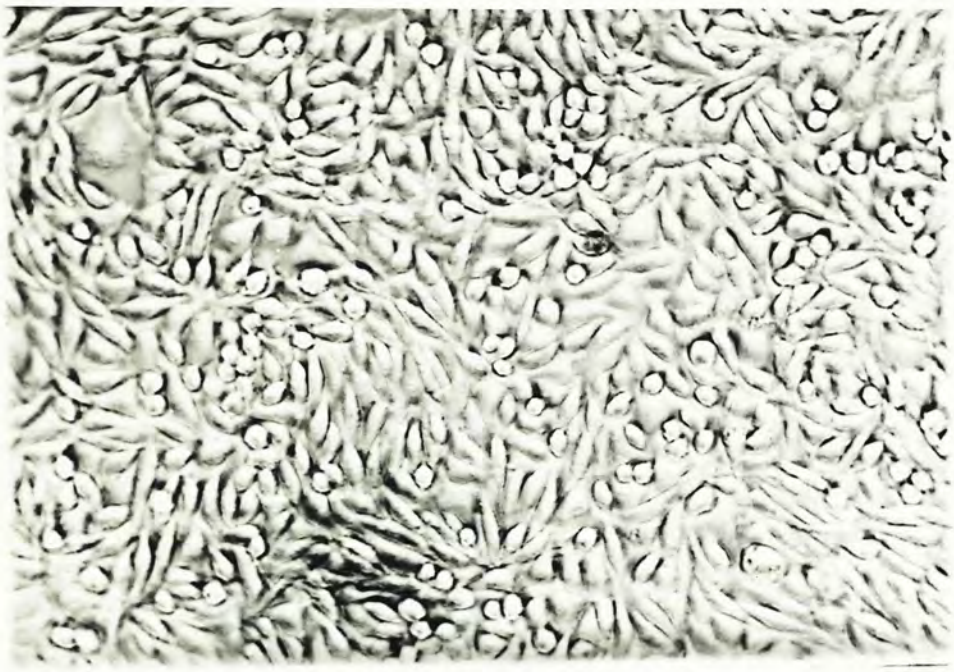


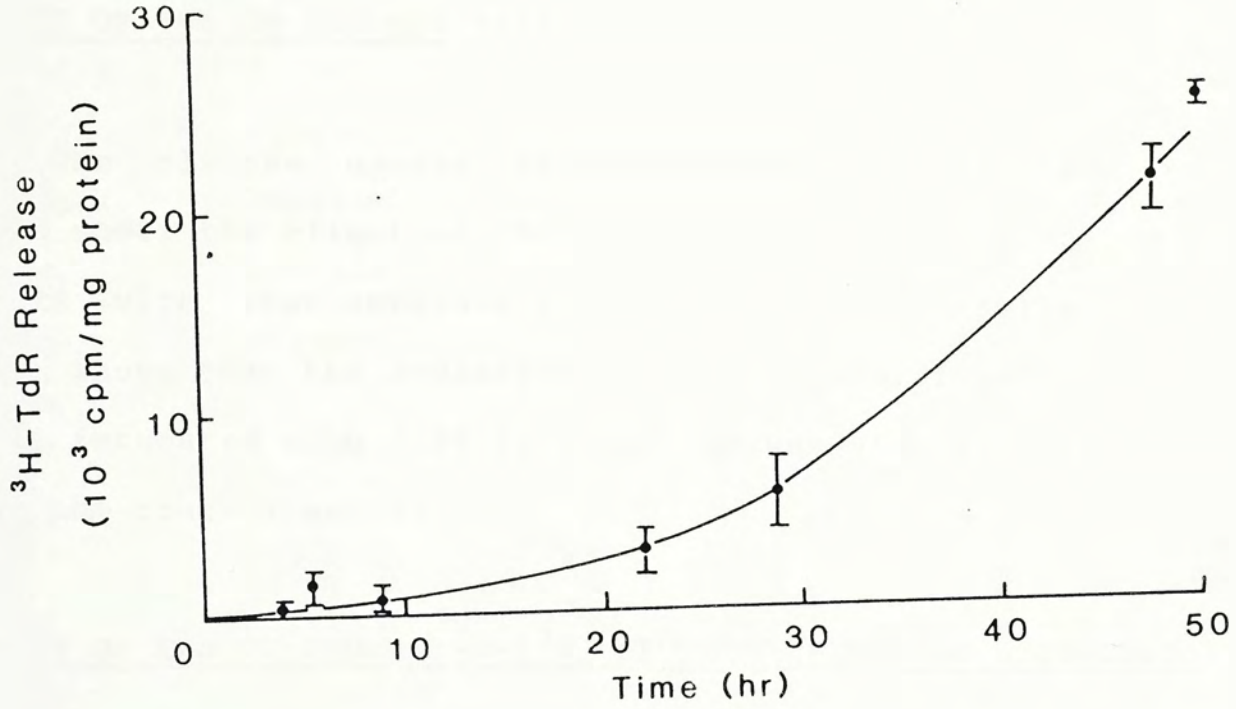
Fig. 6.1B

Fig. 6.1C

Fig. 6.1D

Fig. 6.2 Effect of C. parvum-TNS on L929 cells.
Cells were incubated in RPMI complete medium supplemented with 0.5% (v/v) TNS. [^{3}H]-Thymidine release was monitored as described in the text. Values are presented as mean ± S.E.M. of triplicate determinations.

Fig. 6.2


30
20
10
0
10
20
30
40
50
Time (hr)
^{3}H-TdR Release
(10^3 cpm/mg protein)

hours. Fig 6.3 shows the [^{3}H]-thymidine release assay of the above four groups (in Fig. 6.1). The results show that for the control, cycloheximide and TNS groups, there is no significant release of [^{3}H]-thymidine before 24 hours. However, the release of [^{3}H]-thymidine in TNS + cycloheximide group was significantly higher than other groups.

EFFECT OF TNS ON GLUCOSE TRANSPORT OF EAT CELLS

The glucose uptake of cultured Ehrlich ascites tumor cells under the effect of TNS were carried out to compare the result with that obtained by L cells. The results (Table 6.1) shows that the 2-deoxy-D-glucose uptake of cultured EAT cells incubated with 0.5% (v/v) of TNS was significantly lower than the control serum.

EFFECT OF TNS ON 2-DEOXY-D-[^{3}H]-GLUCOSE UPTAKE OF L929-CELLS

The 2-deoxy-D-glucose uptake of cultured L cells was carried out as decribed in Chapter 3. Fig. 6.4 shows the result of a typical experiment. Since the conventional cytochalasin B binding method (Chan *et al.*, 1983) cannot be used in L929 cells (see Appendix I), the extent of the diffusion-mediated uptake can be estimated graphically by drawing a line through the origin and parallel to the linear portion of the total uptake curve (Renner, Plagemann & Bernlohr, 1972; Plagemann & Richey, 1974). The uptake rate

due to the transport reaction was obtained by subtracting the estimated diffusion rate due to the unsaturable component from the overall rate. The corrected rates showed the Michaelis type kinetics.

Fig. 6.5 shows the time kinetics of 2-deoxy-D-[^{3}H]-glucose uptake of L929 cells incubated with RPMI medium supplemented with or without 2% control serum respectively. Control serum was obtained by injecting 25 ug/ml LPS to mice. There was no great difference for the time-profile of cells incubating in media with or without control serum incubation. This indicates that control serum will not affect the glucose transport experiments.

Fig. 6.6 shows a typical experiment on the determination of glucose transport rate of C. parvum-induced TNS treated L929 cells and Table 6.2 shows the kinetic parameters of 2-deoxy-D-glucose uptake for control and these TNS treated cells. The maximal 2-deoxy-D-glucose uptake rate (Vmax) of the C. parvum treated group shows a two-fold increase of Vmax value comparing with control (Fig. 6.6 and Table 6.2). The apparent half-saturation constant for the specific transport process, Km, of TNS group exhibited small changes (Table 6.2).

EFFECT OF TNS ON PROTEIN CONTENT IN L929 CELLS

Since the 2-deoxy-D-glucose uptake of L929 cells was expressed in terms of nmol/mg protein instead of cell number,

an experiment was carried out to examine the protein content of L cells incubated for 24 hours in medium, or medium supplemented with 2% LPS (control) serum, or with 2% *C. parvum*. induced TNS. The results (Table 6.3) show that there was no difference between protein content of TNS treated cells and cells from control serum or medium groups. The increase of uptake rate of glucose in TNS-treated cells are not due to the decrease of protein content after treatment.

COMPARISON OF TNS FROM DIFFERENT PRIMING AGENTS ON GLUCOSE UPTAKE ACTIVITY OF L929 CELLS

The effects of TNS produced from different priming agents, namely zymosan, *Listeria* and *Mycobacterium lepraemurium* (MLM), on the 2-deoxy-D-[^{3}H]-glucose uptake activity of L929 cells were compared (Table 6.4). Except MLM-TNS, TNS produced from other two priming agents could increase significantly the Vmax value of uptake activity ($P < 0.001$). However, all preparations of TNS did not affect greatly the Km value of the uptake process. It should be noted that TNS induced by *C. parvum* (Table 6.2), zymosan and *Listeria* (Table 6.4) could exhibit about two-fold increases on glucose transport rate (Vmax) of cultured L929 cells. Both TNS have little effect on the nature of transport (expressed by Km values) of the cells.

Fig. 6.3 Time kinetics of cytolytic effect of media complemented with 2% C. parvum-TNS (●——●) or 2.5 ug/ml cycloheximide (▲——▲) or 2% C. parvum-TNS + 2.5 ug/ml cycloheximide (✪——✪). [^{3}H]-Thymidine release was monitored as described in the text.

Values are presented as mean ± S.E.M. of quadruplicate determinations.

Fig. 6.3

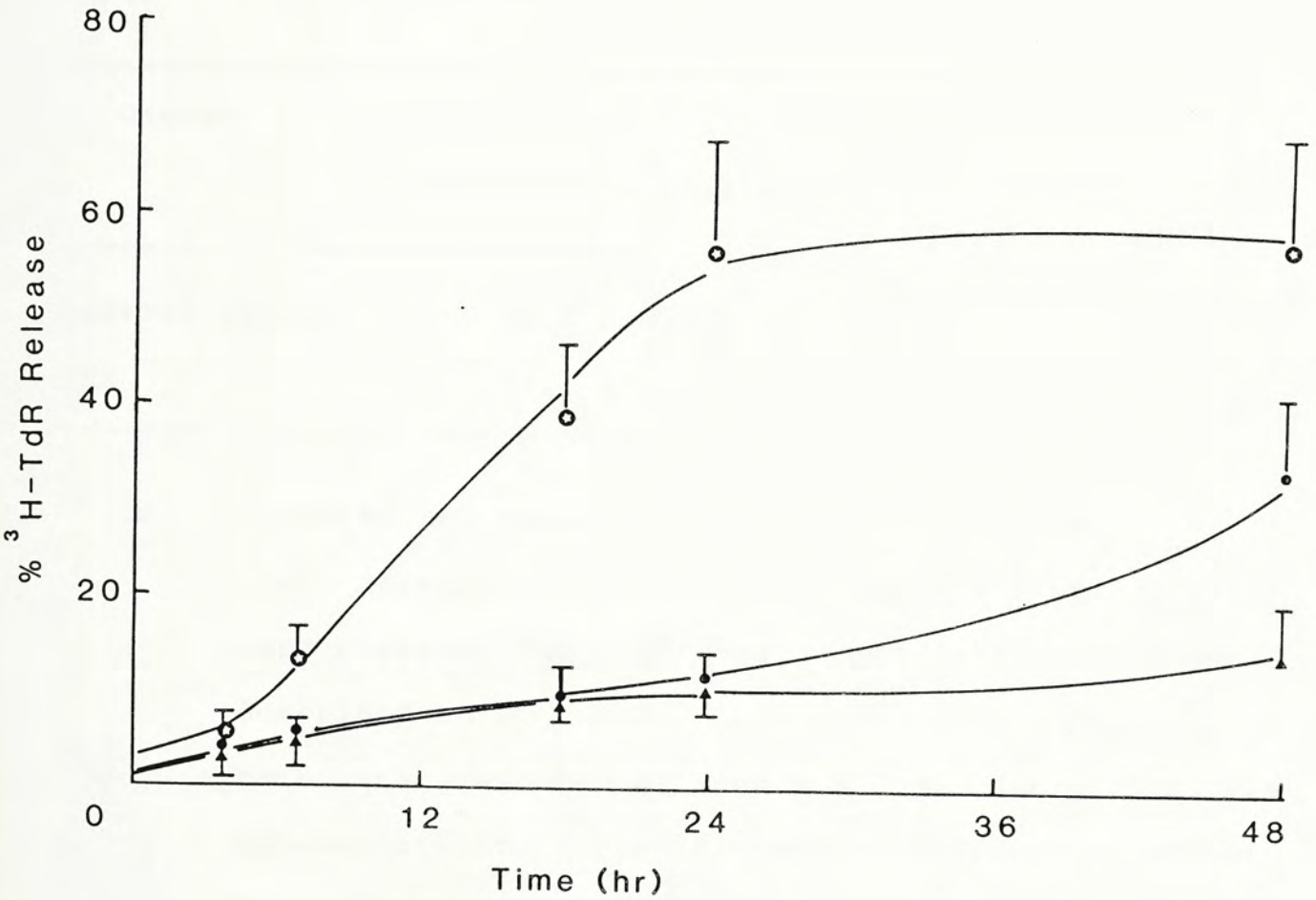


Table 6.1 Effect of TNF on glucose uptake activity of Ehrlich ascites tumor cells

Groups	Vmax ($nmol/min/10^6$ cells)	Km (mM)
Control serum	30.7 ± 3.1	1.16 ± 0.3
TNS	11.0 ± 2.6*	1.14 ± 0.2

a Cultured EAT cells were incubated with 0.5% (v/v) of the appropriate serum at 37 °C. Harvested cells were examined for their ability to take up glucose as described in the text.

b Data are presented as mean ± S.E.M. for triplicate determinations. Significance of difference between values of control and treated cells was determined by studnet's t-test.

* $P < 0.005$

Fig. 6.4 The determination of uptake rate due to the transport reaction in L929 cells.

The curve for total uptake rate against various concentrations of 2-deoxy-D-glucose was plotted (●——●). The extent of diffusion-mediated uptake can be estimated graphically by drawing a line through the origin and parallel to the linear portion of the uptake curve. The uptake rate due to the transport reaction (○——○) was obtained by subtracting the estimated diffusion rate due to unsaturable component from the total uptake rate.

Fig. 6.4

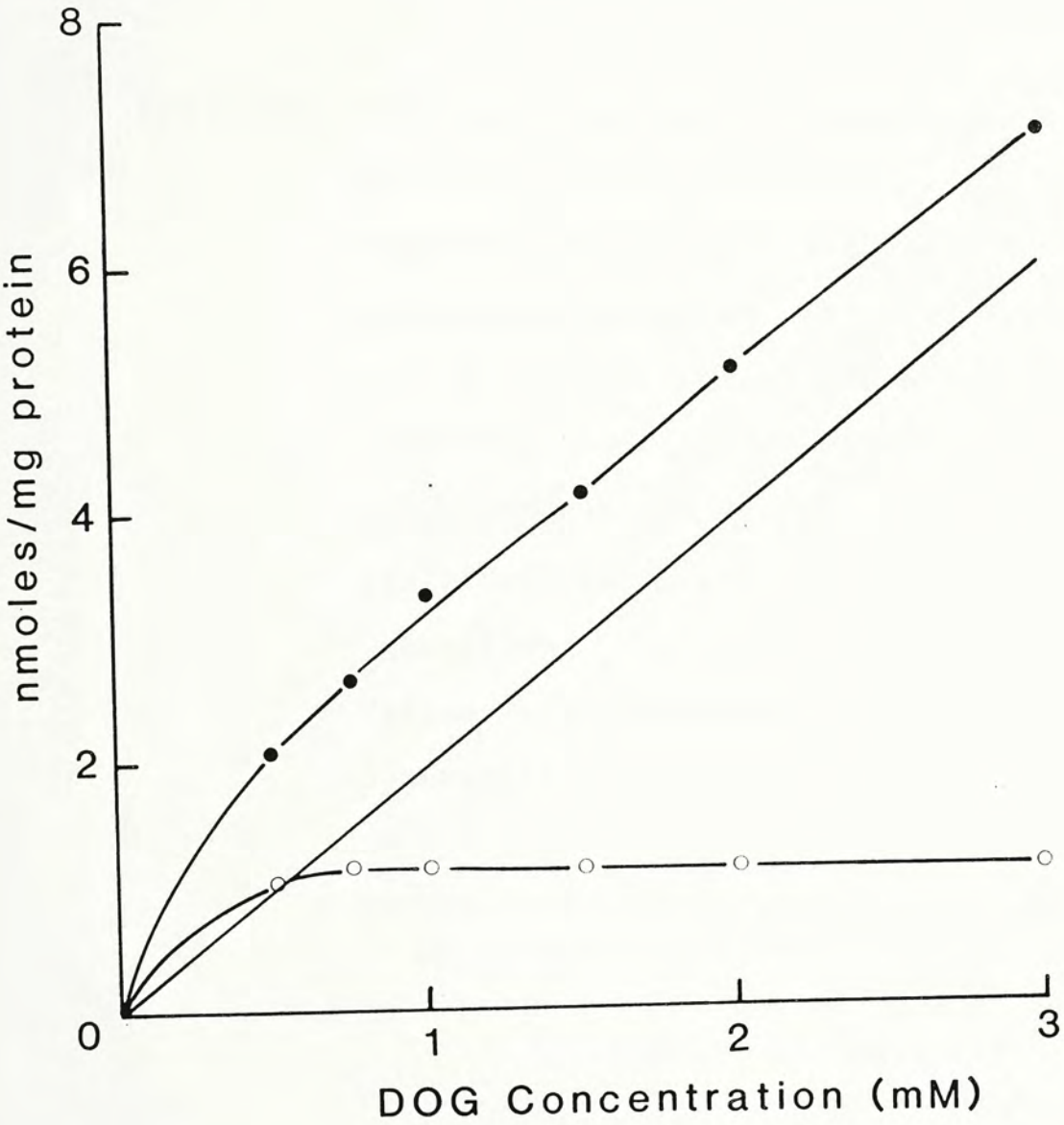


Fig. 6.5 The time kinetics of 2-deoxy-D-glucose uptake of L929 cells incubated with RPMI medium supplemented with or without control serum. Cells were incubated with RPMI medium (●—●) or 2% (v/v) control serum (serum of mice receiving LPS only) (○—○) for 24 hours. 2-deoxy-D-glucose uptakes of both groups were performed at the indicated times of incubation.

Values are presented as mean of triplicate determinations.

Fig. 6.5

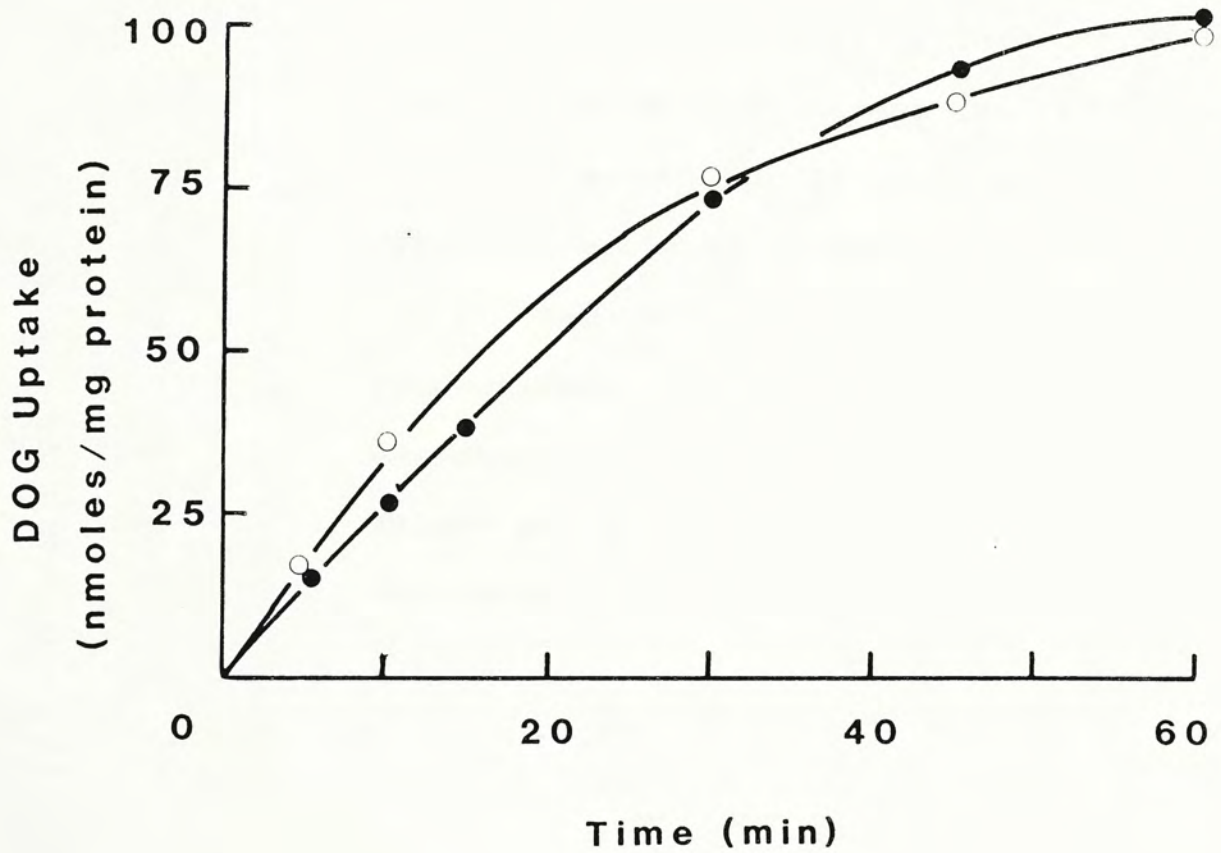


Fig. 6.6 The time kinetics of 2-deoxy-D-glucose uptake of L929 cells incubated in RPMI media supplemented with control serum or C. parvum-induced TNS.

Cells were incubated with control serum (2% v/v) (●—●), C. parvum-induced serum (2% v/v) (▲—▲) for 24 hours at 37 C. The diffusion-mediated uptakes are presented as (○) and (△) for control serum and TNS respectively. The uptake of 2-deoxy-D-glucose was measured for the indicated time intervals. Values are presented as mean of quadruplicate determinations.

Fig. 6.6

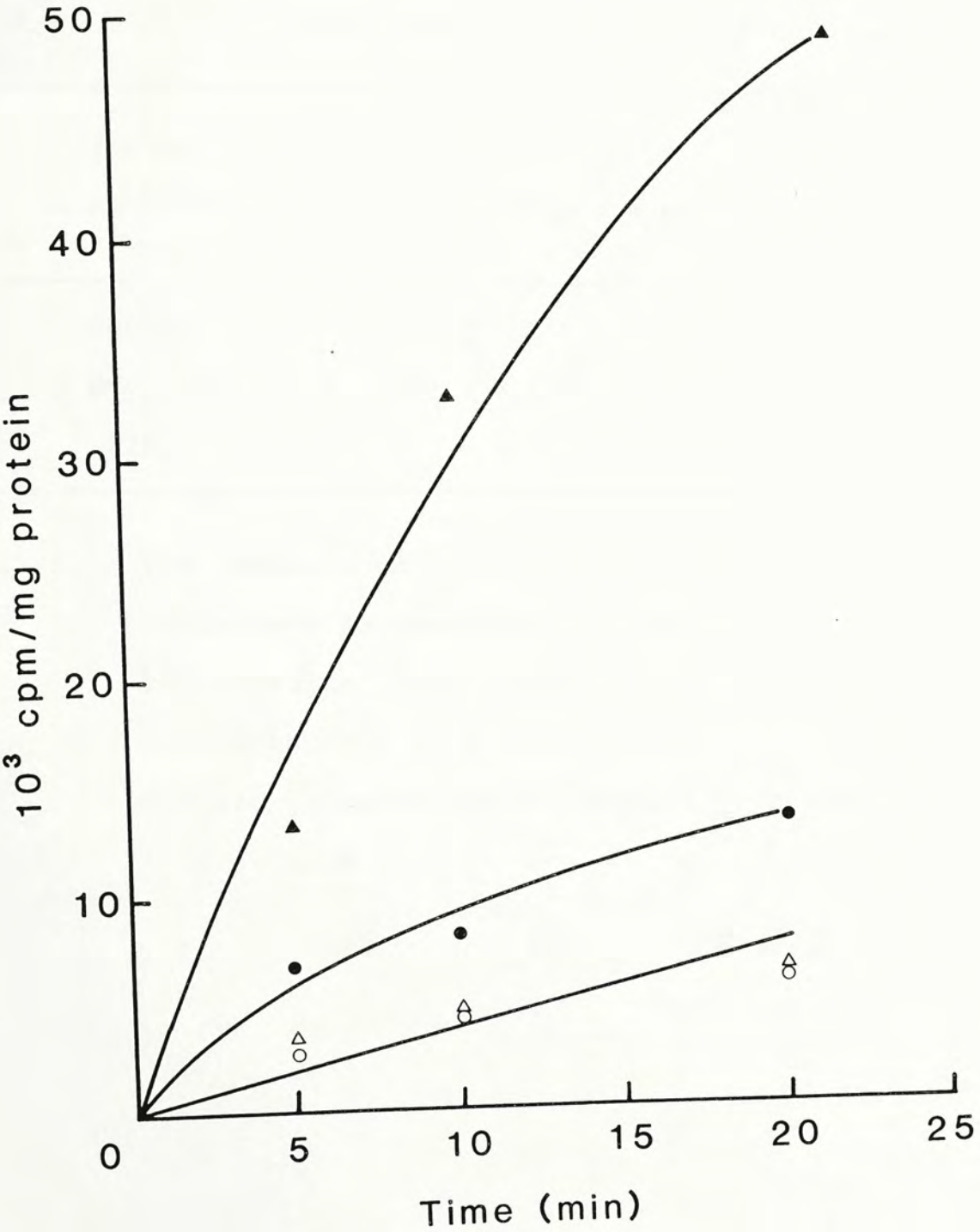


Table 6.2 The effects of C. parvum induced TNS on 2-deoxy-D-glucose uptake of L929 cells.[a]

Groups	Vmax (nmol/min/mg protein)	Km (mM)
Control	1.18 ± 0.31	0.11 ± 0.08
C. parvum-TNS (2%)	2.24 ± 0.56*	0.50 ± 0.20

a The details of 2-deoxy-D-[^{3}H]-glucose uptake of L929 cells were as described in the text.

The results are presented as mean ± S.E.M. of determinations of 2 experiments.

The significance was determined by Student's t-test.

* $P < 0.005$

Table 6.3 The effect of TNS on the protein content of L929 cells.[a]

Supplement in RPMI Medium	Protein content per cell (ng protein / cell)
None	0.875 ± 0.158
Control Serum (2% LPS Serum)	0.909 ± 0.172[#]
C. parvum-TNS (2%)	0.915 ± 0.114[#]

a The procedures are as described in the text.
The data are presented as mean ± S.E.M. of 6 determinations.
The significance of difference was determined by Student's t-test.

$P > 0.1$ as compared with results obtained from cells incubated with RPMI medium only.

Table 6.4 The effect of TNS from different priming agents on the uptake kinetics of 2-deoxy-D-glucose of L929 cells.[a]

Groups	Vmax (nmol/min/mg protein)	Km (mM)
Control	3.25 ± 0.47	0.18 ± 0.06
Zymosan-TNS	6.90 ± 0.43*	0.38 ± 0.04
Listeria-TNS	6.07 ± 0.48*	0.70 ± 0.05
MLM-TNS	3.83 + 0.20#	0.21 ± 0.05

a TNS induced by zymosan, Listeria, and Mycobacterium lepraemurium (MLM) were prepared as described in Experimental section in the text.

The details of 2-deoxy-D-[^{3}H]-glucose uptake of L929 cells were as described in the text.

The results are presented as mean ± S.E.M. of quadruplicate determinations.

* $P < 0.001$

\# $P > 0.05$

DISCUSSION

L929 cells have been reported to be very sensitive to TNS (Carswell et al., 1975; Matthews & Watkins, 1978) and has been used for in vitro TNF assay. From our results (Fig. 6.1) we can find that TNF is effective against the viability of L cells. It has been reported that TNS cytotoxicity, measured by the release of [^{3}H]-thymidine or ^{51}Cr from labeled L929 cells, could be detected only after 8 - 12 hours of incubation (Ruff & Gifford, 1981b; Matthews & Watkins, 1978). Our results (Fig. 6.2) also support this suggestion. Our results also demonstrated that combined use of TNS and cycloheximide can enhance the cytotoxic effect of TNF (Fig. 6.1C). The cells after incubating with medium supplemented with 1.25% (v/v) TNS and 2.5 ug/ml cycloheximide for 24 hours, have lost their integrity and rounded up to death (Fig. 6.1C). The cytotoxic effect of TNS in the presence of cycloheximide was further proved by the [^{3}H]-thymidine release assay. As indicated in Fig. 6.3, while less than 10% of [^{3}H]-thymidine were released from TNS-treated or cycloheximide-treated cells as compared with control in 24 hours incubation, nearly 55% [^{3}H]-thymidine release was observed in TNS + cycloheximide treated cells after 24 hours incubation. The findings that the anti-tumor effect of TNS in vitro can be enhanced in the presence of cycloheximide suggests a more sensitive method for the estimation of TNS titer by using L cells. It is also of interest to examine whether this synergistic effect of TNF and cycloheximide on tumor cells also appears in experimental

animals.

In EAT cells, the treatment of C. parvum-induced TNS can inhibit the uptake of glucose significantly (Table 6.1 and Fung et al., 1985). In contrast, the uptake of 2-deoxy-D-glucose increases in C. parvum-induced TNS treated L cells (Table 6.2). This discrepancy is difficult to explain. One possible explanation is that TNF induced by C. parvum might have different actions on EAT cells and L cells. In order to test whether there is any contaminate in the serum that might affect the glucose carrier of L cells, we examined the glucose uptake profile of L cells incubating in plain RPMI medium and medium supplemented with control serum (produced by LPS injection only). Fig. 6.5 indicats that the glucose uptake in both situations were in similar profile. To further study whether the increase in Vmax values of L cells is unique in C. parvum-induced TNF, we also tested TNFs produced from different sources. Table 6.4 shows that both Listeria-induced and zymosan-induced TNS could increase Vmax of glucose uptake in L cells. It is also interesting to note that the increases of Vmax in L cells after C. parvum-, zymosan- and Listeria-induced TNF's treatments are relatively similar, with the range at about 2 folds.

Matthews (1983) has reported that the treatment of L cells with cytotoxin from human monocytes can decrease the respiration rate and increase the rate of glycolysis and glucose uptake in the tumor cells. The mitochondrial

dysfunction after cytotoxin treatment might cause the increased rate of glucose utpake and glycolysis which is considered to be a compensatory action of the tumor cells to counteract this stress. We do not know the action mechanism of TNF, and we have not measured the respiration rate of L cells and therefore cannot be sure the same change has happened in the L cells after the incubation with TNS.

Our observations that the binding of cytochalasin B is not displaceable by D-glucose (Appendix I) suggests the difference between the glucose uptake properties of L929 cells and Ehrlich ascites tumor cells. This kind of lack of inhibition of cytochalasin B binding by glucose as observed in L929 cells, also occurs in macrophages and lymphocytes (see Appendix I). We may conclude by saying that the glucose uptake properties and the nature of cytochalasin B binding are not universal for all kinds of cells. The difference between glucose uptake activities in L cells and Ehrlich ascites tumor cells is at best one of the examples. However, we do not know the action mechanism of TNS on the tumor cells. The difference of the glucose uptake activity in L cells and Ehrlich ascites tumor cells may just be the manifestation of different action of TNS on the cells. In appendix I, we studied the effect of TNS on the permeability of membrane of L cells. Results indicate that TNF treated L cells also exhibitded increases of uridine and Ca uptake. We are now in the progress to study whether similar changes would occur in TNS-treated Ehrlich ascites tumor cells.

CHAPTER SEVEN

THE EFFECT OF ANTIMETABOLITES ON THE GLUCOSE TRANSPORT OF L929 AND EHRLICH ASCITES TUMOR CELLS

INTRODUCTION

There are many antitumor agents which have been extensively studied for their cytotoxic effects. These antitumor agents include methotrexate (Hryniuk, 1972; Kaminskas & Nussey, 1978; Ernst, 1971; Lala & Patt, 1966), N-(phosphonacetyl)-L-aspartate (PALA) (Baillon et al., 1983; Plageman & Richey, 1974; Tsuboi & Kwong, 1978; Collins & Stark, 1971), tumor necrosis factor (TNF) (Matthews, 1983; Carswell et al., 1975; Matthews & Watkins, 1978; Green et al., 1977; Ha et al., 1985; Fung et al., 1985), interferon and interferon inducers (Kleinschmidt, 1972; Gresser et al., 1970; Nagano & Takano, 1982; DuBy, 1972; DeClercq, 1981; Stringfellow, 1981; Riviere & Hovanessian, 1984). Most of these antitumor agents are thought to have inhibitory effects on DNA, RNA and protein synthesis of tumor cells and hence exhibit the tumor inhibitory effects.

The tumor models we employed are L929 and Ehrlich ascites tumor cells. Since Ehrlich ascites tumor cells depend primarily on glycolysis for the provision of energy and to maintain growth potential, it is essential that they possess a

highly efficient system for glucose transport and metabolism (Chan et al., 1983; Leung et al., 1984; Fung et al., 1985). Since the carrier mediating the glucose transport is a protein (Cuppoletti, Mayhew & Jung, 1981), a hypothesis has been raised by us during our studies of methotrexate, PALA and TNF on Ehrlich ascites tumor cells that the depletion of nucleotides by these antitumor drugs and the consequent inhibition of DNA, RNA and protein synthesis might have constituted the primary chain of events leading to reduced carrier production. In order to prove this hypothesis, we studied the effects of two commonly used antimetabolites, namely cycloheximide and actinomycin D on the glucose transport of tumor cells. Cycloheximide has an inhibitory effect on protein synthesis in cytosol ribosomes, whereas actinomycin D inhibits RNA synthesis by intercalating between DNA bases, especially in G-C rich regions. It is conceivable that they might have certain effect on glucose carrier synthesis during tumor cell growth.

AIM OF STUDY

In this chapter, the experiments of the effects of cycloheximide and actinomycin D on glucose transport activities of L929 and Ehrlich ascites tumor cells are described. The effects of both antimetabolites on the precursors incorporation of EAT cells are also discussed.

EXPERIMENTAL

PREPARATION OF DRUG SOLUTION

Cycloheximide and actinomycin D were dissolved in PBS (pH 7.4) at concentration of 0.45 mg/ml. The reconstituted solution was stored in freezer until use. The solution was diluted to the desired concentrations with PBS and used directly for injection.

TREATMENT OF TUMOR BEARING MICE

Mice were inoculated i.p. with 2×10^6 Ehrlich ascites tumor cells harvested from 7-day old tumors in 0.2 ml PBS on day 0. The effects of cycloheximide and actinomycin D were observed in groups of at least 10 mice each. In the test groups, cycloheximide and actinomycin D (0.9 ug/mouse or 1.8 ug/mouse) were administered intraperitoneally in 0.2 ml PBS on days 2, 4 and 6 inclusively. The mice were sacrified by cervical dislocation on day 7. Tumor cells were washed 5 times with half-isotonic saline to remove blood cells and harvested by centrifugation. The final cell suspension was prepared in PBS. Cells were counted with a haemocytometer and resuspended in the same buffer to 2×10^7 cells/ml. Glucose uptake and cytochalasin B binding were then measured as described in Chapter 3. For the determinations of incorporation of leucine, uridine and thymidine, the cells were resuspended in PBS to 10^6 cells/ml and the methods as

described in Chapter 3 were followed.

TREATMENT OF L929 CELLS

10^5 L929 cells were incubated in 0.5 ml complete RPMI 1640 medium with 2.5 ug/ml cycloheximde or complete medium at 37 °C for 24 hours. Then the glucose uptake experiment was carried out as described in Chapter 3 and Chapter 6.

RESULTS

THE EFFECTS OF ANTIMETABOLITES ON TUMOR SIZE OF EAT CELLS

The effects of cycloheximide and actinomycin D on tumor size of Ehrlich ascites tumor in tumor-carrying mice were studied (Table 7.1). The results show that cycloheximide is not effective in reducing tumor size whereas actinomycin D can reduce the tumor size by 20% ($P < 0.01$) at dose 0.9 ug/mouse. When the dose is raised to 1.8 ug/mouse, both antimetabolites are effective in reducing EAT tumor size. Cycloheximide can reduce the tumor size by 23% ($P < 0.05$), whereas actinomycin D can reduce the tumor size by 70% ($P < 0.001$).

EFFECTS OF ANTIMETABOLITES ON THE GLUCOSE TRANSPORT OF EAT CELLS

Both the 2-deoxy-D-[^{3}H]-glucose uptake and cytochalasin B binding were carried out to assay the effects of cycloheximide and actinomycin D on the glucose transport activity of Ehrlich ascites tumor cells. The results (Table 7.2) show that actinomycin D at dose 1.8 ug/mouse was effective in decreasing both 2-deoxy-D-[^{3}H]-glucose maximal uptake rate (Vmax) and glucose-sensitive cytochalasin B binding (Bo). The Vmax value was significantly reduced by half ($P < 0.02$) and the Bo value reduced by 65% ($P < 0.001$). The Km and Kd values showed no great change in values. However, cycloheximide at dose 1.8

ug/mouse did not show any significant reduction in Vmax and Bo values ($P > 0.02$).

EFFECTS OF ANTIMETABOLITES ON PRECURSORS INCORPORATION IN EAT CELLS

Cycloheximide group (1.8 ug/mouse) showed a reduction in leucine incorporation ($P < 0.001$), suggesting that protein synthesis was inhibited. However, the incorporation of thymidine and uridine were increased. The actinomycin D group (1.8 ug/mouse) showed a significantly great reduction in the incorporation of leucine (60%), thymidine (50%) and uridine (30%).

EFFECT OF CYCLOHEXIMIDE ON 2-DEOXY-D-[^{3}H]-GLUCOSE UPTAKE OF L929 CELLS

The results shown in Table 7.4 indicate that 2.5 ug/ml cycloheximide did not show any effect on the glucose uptake rate ($P < 0.1$) of the L cells culture and the Km values of control culture and cycloheximide treated culture did not show any great difference.

Table 7.1 The *in vivo* effects of both antimetabolites on tumor growth of EAT cells.[a]

Treatment	Tumor size (% of Control)
Cycloheximide (0.9 ug/mouse)	100.22 ± 23.06[#]
Cycloheximide (1.8 ug/mouse)	77.69 ± 27.20[*]
Actinomycin D (0.9 ug/mouse)	80.14 ± 13.74[**]
Actinomycin D (1.8 ug/mouse)	31.65 ± 8.95[***]

a The details of experiment are as described in the text.

The significance of difference between test and control groups was determined by Student's t-test.

Significant differences: *** $P < 0.001$

** $P < 0.01$

* $P < 0.05$

$P > 0.2$

as compared with control.

Table 7.2 The *in vivo* effects of antimetabolites on glucose transport activity of EAT cells.[a]

Groups	2-deoxy-D-glucose uptake		Cytochalasin B binding	
	Vmax ($nmol/min/10^6$ cells)	Km (mM)	Bo ($pmol/10^7$ cells)	Kd (10^{-7} M)
Control	16.19±2.59	0.87±0.21	75.26±5.4	1.76±0.26
Cycloheximide (1.8 ug/mouse)	14.13±1.07[#]	0.78±0.30	72.73±4.1[#]	1.78±0.25
Actinomycin D (1.8 ug/mouse)	8.60±2.07[**]	1.09±0.39	26.16±3.2[*]	1.27±0.30

a The details of experiment are as described in the text.

Significant differences: * $P < 0.001$

** $P < 0.02$

\# not significant

as compared with control.

Table 7.3 The *in vivo* effects of antimetabolites on precurors incorporation in EAT cells.[a]

Treatment	% Incorporation[b]		
	Leucine	Thymidine	Uridine
Cycloheximide (1.8 ug/mouse)	81.78 ± 6.78***	127.01 ± 20.92*	146.36 ± 40.53**
Actinomycin D (1.8 ug/mouse)	43.25 ±16.00***	49.13 ± 34.07**	70.52 ± 29.09#

a The details of experiment are described in the text.

b Assuming the incorporation of control group is 100%

Significant differences: *** $P < 0.001$

** $P < 0.02$

* $P < 0.05$

$P > 0.05$

as compared with control.

Table 7.4 *In vitro* effect of 2.5 ug/ml cycloheximide on the 2-deoxy-D-glucose uptake of L929 cells[a].

Groups	2-deoxy-D-glucose uptake	
	Vmax (nmol/min/mg protein)	Km (mM)
Control	1.18 ± 0.31	0.11 ± 0.08
Cycloheximide	1.11 ± 0.65#	0.24 ± 0.04

a The details of the experiment are as described in the text.

Significant difference: # not significant, as compared with control.

DISCUSSION

It has been postulated that the depletion of macromolecules necessary for the production of glucose carrier would lead to defects in glucose transport activity of Ehrlich ascites tumor cells (Chan et al., 1983; Leung et al., 1984; Fung et al., 1985). This conclusion has further proved by the results of the present study. Actinomycin D which inhibits RNA synthesis by intercalating the DNA bases has a drastic effect of suppressing both tumor size and glucose transport activity of EAT cells (Table 7.1 and 7.2). The reduction of tumor size paralleled the reduction in both the maximal uptake rate and glucose-sensitive cytochalasin B binding. Since only the Vmax and Bo values were reduced and no significant changes in Km and Kd values, we suggest that the destructive effect of actinomycin D is on the quantities of carrier on the cell membrane. While alteration in the turnover rate of the glucose carriers cannot be absolutely ruled out in the present study, its contribution to the overall transport process is at best minimal. The results of precursors incorporation also support this conclusion (Table 7.3). The incorporation of all precursors have been significantly reduced in actinomycin D treated EAT cells and this suggests that DNA, RNA and protein synthesis have been impaired. The defects of protein synthesis would consequently affect the number of glucose carriers and then the glucose uptake. The result of cycloheximide may show that the inhibition of protein

synthesis have not been completed. The reduction of leucine incorporation is only 20% and there are no reductions in other precursors incorporation (Table 7.3). The lack of inhibition of the dose of cycloheximide (1.8 ug/mouse) under test on the glucose transport activities (Table 7.2) can also be compared with the small effect of the same dose of drug on tumor growth (Table 7.1)

The minimal antitumor effect of cycloheximide observed in Ehrlich ascites tumor bearing mice was re-examined in vitro with another tumor line, L929. The treatment with 2.5 ug/ml cycloheximide is also ineffective in decreasing the glucose uptake of cultured L929 cells.

From the results of above experiments, we may conclude that the effect of actinomycin D on the glucose transport process in Ehrlich ascites tumor cells is due to the reduction in glucose carrier number. This effect parallels with the antitumor effect of actinomycin D on the tumor size of EAT. The small antitumor effect of cycloheximide on tumor size may be due to its lack of efficacy in affecting the glucose transport system in Ehrlich ascites tumor. Similar effect has been obtained by the treatment of cycloheximide on L929 cells. It is also conceivable that suppression of RNA synthesis is an important mechanism of anti-tumor agents which can inhibit tumor growth by reducing glucose carriers of the tumor cells.

CHAPTER EIGHT

GENERAL CONCLUSION AND OUTLOOK

The effects of some anticancer agents on the glucose transport system, especially on the glucose uptake of L929 and Ehrlich ascites tumor cells were investigated in this thesis.

EFFECTS OF THYMIDINE AND URIDINE RESCUE ON METHOTREXATE-TREATED EHRLICH ASCITES TUMOR CELLS

The effect of methotrexate on glucose carrier in Ehrlich ascites tumor cells has been studied in our previous work (Chan *et al.*, 1983). It has been proposed that the direct effect of methotrexate is on the inhibition of nucleotide synthesis through the impairment of folate system and induction of a "purineless" state in cells (Hryniuk, 1973). To further investigate the underlying action mechanism of methotrexate and to examine the effectiveness of exogenous thymidine rescue for tumor cells, we studied the reversal effect of thymidine in methotrexate treated Ehrlich ascites tumor cells. The results show that the tumor cells, after thymidine administration *in vivo* and *in vitro*, can partially recover the growth capability, glucose uptake rate and glucose carrier number to the status before the methotrexate treatment. Hoglind-Semon and Grindey (1978) suggested that the mechanism if thymidine rescue for normal cells is through

the metabolic conversion of thymidine to thymidine-5'-triphosphate and decreased rate of thymidylate synthetase. The decreased rate of thymidylate synthetase would not deplete the tetrahydrofolte pool whose production has already been inhibited by methotrexate. We postulated that this rescue mechanism appearing in normal cells might also take place in tumor cells. Since Ehrlich ascites tumor cells have a comparatively large number of glucose carriers on their membrane than other cells (Chan et al., 1983) and these glucose carriers are protein in nature (Cuppoletti, Mayhew and Jung, 1981), the inhibition of methotrexate on nucleotide and the consequent inhibition of DNA, RNA and protein synthesis may have constituted primary chain of events leading to reduced glucose carrier production (Chan et al., 1983). The protection of exogenous thymidine on nucleotide synthesis would therefore relieve the chain of inhibition. This suggestion is supported by the results of our experiment that the rescue of thymidine of tumor cells are accompanied by partial reversal of methotrexate on the maximal glucose uptake rate (Vmax) and glucose-sensitive cytochalasin B binding (Bo), without affecting the Michaelis constant (Km) of glucose uptake and apparent dissociation constant of glucose-sensitive cytochalasin B binding (Kd), of the tumor cells.

The ineffectiveness of exogenous uridine in methotrexate rescue on tumor cells might be due to the production of deoxyuridine-5'-monophosphate by the cells through the salvage

process and aggrevate the impaired folate system by enhancing the reaction of thymidylate synthetase. The possible rescue of tumor cells by the administration of purine or pyrimidine or their analogs and their effects on glucose transport of tumor cells are also worth studying.

EFFECTS OF INTERFERON INDUCERS ON EAT CELLS

The results of interferon inducers also support our suggestion of primary chain of action of anticancer agents on glucose carrier of Ehrlich ascites tumor cells. The administration of Poly I:C, tilorone and statolon can effectively reduce the tumor size and also the number of glucose carrier and the uptake rate. However, information about the action mechanism of these interferon inducers is still lacking. The interferon titer in the sera of treated mice we obtained 24 hours after administration of inducers was quite low. The production of interferon might have already been inhibited by the immune system of the outbred mice. Therefore we suggested the use of inbred mice in the experiment and the earlier collection of serum (2-4 hours after the administration of inducers) might minimize the degradation and hyporeactivity effects.

The admininstration of interferon inducers (i.p.) near the tumor site (i.p.) is more effective in reducing the tumor size than other routes of administration. Similar results have been obtained by DuBuy (1972) and Gresser & Bourali

(1970). The difference of effectiveness might be due to the direct effect of local interferon induced or the direct effect of the inducers on the tumor. It has been postulated that the antitumor effects of inducers like Poly I:C do include direct chemotherapeutic effect or host-mediated reaction besides the action of interferon induced (Talal, 1971; Levy et al., 1970).

The effectiveness of Poly I:C treatment before the inoculation of tumor is a promising direction for the discovery of a low toxic precautionary drug against some spontaneous high-risk tumor. The DEAE:Poly I:C Complex and the Poly (ICLC) developed which possess greater quantity of interferon production and higher resistance of enzymatic degradation may be a promise to this direction (Pitha & Carter, 1970; DeClercq, 1981). Should these highly efficient interferon inducers have more pronounced effect of the glucose transport of tumor cells is also of interest to investigate.

EFFECT OF TNS ON GLUCOSE UPTAKE OF L929 CELLS

The effects of TNS produced by different priming agents show an increase in 2-deoxy-D-glucose uptake rate of L929 cells. Since the effect of TNS on L cells observed by Darzynkiewicz et al. (1984) is the initial cytostatic and the later cytolytic response, the increased glucose uptake rate might be responsible for the increased division rate and the consequent death of the cells after a long time of incubation with TNS. However, the underlying action mechanism of TNS is

still obscure. The contrasting results of the effect of TNS on Ehrlich ascites tumor (Fung et al., 1985) may suggest different responses of tumor cells to TNS or the nature of glucose carrier of these two tumor cells might be different.

EFFECT OF ANTIMETABOLITES ON L929 AND EAT CELLS

The antitumor effects of cycloheximide and actinomycin D were investigated. Actinomycin D showed an inhibitory effect on tumor growth, glucose uptake and carrier number of Ehrlich ascites tumor cells. The conclusion is similar to that purposed by us in our previous work that the inhibition of macromolecules synthesis would lead to impairment of protein synthesis and consequently the reduction of glucose carrier number in Ehrlich ascites tumor cells. The mechanism concerning the inability of cycloheximide in arresting tumor growth and glucose uptake rate in L929 and Ehrlich ascites tumor cells awaits further examination.

SCOPE OF FURTHER STUDY

In the present study, we have shown that methotrexate, interferon inducers and tumor necrosis factor are effective in suppressing Ehrlich ascites tumor growth and reducing glucose carrier number. Our previous studies have indicated that PALA (Leung et al., 1984), BCNU and CCNU (Leung et al., unpublished data) have similar property. Whether the effect of reduction

of glucose carrier is a primary or secondary reaction of these anti-tumor agents on the glucose carrier protein remains unknown.

In order to test the primary effect of these agents on the glucose carrier of the tumor cells, we plan to purify the glucose carrier of the cell and to test the effects of drugs on the glucose transport status of the liposome composed of the glucose carrier. Concerning the secondary effect of these anti-tumor agents on the glucose transporter of tumor cells, we have demonstrated in this thesis that the inhibition of DNA and/or RNA and/or protein synthesis might be one of the factors. Other factors included: (1) the possible effect of these agents on the translocation of glucose carrier from microsomal pool to the plasma membrane of the tumor cells; (2) the possible effect of these agents on the control of intracellular concentration of purines which have been reported to be a hyper-repressor of glucose carrier in chick fibroblasts (Gay & Amos, 1983); (3) the possible effect of these agents on the control of intracellular concentration of phosphoribosyl diphosphate (PPRibP) has also been reported to be inversely correlated to the glucose transport rate in chick fibroblasts (Gay & Amos, 1983).

Concerning the factor (1), we are now in the progress in studying the possibility of using the photoaffinity labelling with [H]-cytochalasin B (Carter-Su et al., 1982; Shanahan, 1982) to monitor the change of the contents of carriers on

plasma membrane and intracellular fractions. Concerning the factors (2) and (3), we plan to study both the relationship between the intracellular concentrations of purine and PPRibP on the glucose transport of Ehrlich ascites tumor cells and L cells *in vivo* and *in vitro*.

The diversified effects of TNS on Ehrlich ascites tumor cells and L cells are also worth studying. Firstly, we plan to investigate more clearly the nature of the glucose carrier on L929 cells. Secondly, we plan to purify TNF from the sera of mice receivng *C. parvum* and lipopolysaccharide so that no contaminant might take place in our determinations. Thirdly, we will extend our investigation of the effect of TNF to other tumor cell lines with the hope of further understandng of the actual mechanism of TNF on tumor cell growth.

APPENDIX I

GLUCOSE REVERSIBILITY OF CYTOCHALASIN B BINDING IN SOME CELLS

ABSTRACT

Several tumor or normal cell lines were found to have cytochalasin B binding which cannot be displaced by high molar of glucose solution. Among all cell lines tested, binding of cytochalasin B either in the absence or in the presence of 500 mM glucose was the same. Cytochalasin B, thereafter, cannot be used as universal marker for glucose carriers on the surface of cells.

INTRODUCTION

Cytochalasin B is a mold metabolite isolated from Helminthosporium dematioideum. Besides the ralative long term effect on the microfilaments of the cells (Wessells et al., 1971), it was found to be a potent, reversible inhibitor of glucose uptake in many normal cells such as human erythrocytes (Sogin & Hinkle, 1980), adipocytes (Karnieli et al., 1981) and chick embryo fibroblasts (Salter & Weber, 1979) and some tumor cells such as HeLa cells (Lin et al., 1974), Ehrlich ascites tumor cells (Cuppoletti et al., 1981; Chan et al., 1983) and

Novikoff rat hepatoma (Graff et al., 1981). A set of high-affinity glucose-reversible binding sites for cytochalasin B has been identified to be the glucose transporters of these cells. Studies performed on this glucose-reversible cytochalasin B binding activity in the above cell systems reveal that they share a common characteristics: (1) a high affinity for cytochalasin B (Kd values in the range of 1 - 6 x 10^{-7} M); (2) reversibility of binding by 500 mM glucose. Cytochalasin B binding method has now been widely used to estimate the density of glucose carrier of different cells (see Chapter 1 for review).

Most recently, Albert (1984) found that the binding of cytochalasin B and the rabbit erythrocytes could not be displaced by glucose and they claimed that cytochalasin B does not serve as a marker of glucose transporter in rabbit erythrocytes. In our study of glucose transport system of L cells (Chapter 6), we also found that the binding of cytochalasin B on L cells could not be replaced by 500 mM glucose. We report our results on L cells herein and results of some other cells will also be presented.

EXPERIMENTAL

CELL LINES

L929, mice thymocytes, spleen cells and peritoneal macrophages were employed for the test.

L929 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5 mg/ml glucose, 50 units/ml penicillin and 100 ug/ml streptomycin. The medium was buffered with 25 mM HEPES and 25 mM $NaHCO_3$.

The thymocytes and spleen cells were collected from the thymus and spleens of female ICR mice respectively. Tissues were minced and the cells were obtained by pressing through a 50 mesh wire gauze to form a single cell suspension. Blood cells in spleen cell preparations were lysed by incubating the cells in ammonium-tris HCl buffer (pH 7.2) at room temperature for 15 min. Cells were washed three times in PBS before use. Viability of cells always exceeded 95% by trypan blue exclusion test.

Peritoneal macrophages were obtained from female mice injected i.p. with 0.5 mg *C. parvum* 7 days prior to sacrifice. Each animal was lavaged with 10 ml ice-cold saline and cells were pooled from groups of 60 mice. Differential count of

cell suspension using Wright's stain showed that more than 85% of cells were non-specific esterase positive and 80% were phagocytic (Ha et al., 1984). The cells were washed twice in PBS before doing cytochalasin B binding experiment.

CYTOCHALASIN B BINDING OF L929 CELLS

L929 cells which have reached confluency in 24-well plastic tissue culture plate (Falcon) was washed twice with PBS (pH 7.2) and equilibrated to 37 °C. 0.04 uCi [^{3}H]-cytochalasin B and 2 x 10^{-7} M cytochalasin B in volume of 0.5 ml was added to the cells and incubated to the indicated time intervals. For the non-specific binding, 500 mM D-glucose was present in the incubation medium in another plate. The incubation was stopped by aspiration of supernatant and washing with cold saline. Cells were lysed by addition of 0.5 ml 0.1% Triton X-100 to individual well. Radioactivity of the lysate was counted in a Beckman LS-7000 liquid scintillation counter and protein was determined by Lowry's method.

CYTOCHALASIN B BINDING OF THYMOCYTES, SPLEEN CELLS AND PERITONEAL MACROPHAGES

The details of cytochalasin B binding are the same as those described in Chapter 3 for Ehrlich ascites tumor cells.

RESULTS

CYTOCHALASIN B BINDING OF L929 CELLS

The time kinetics of cytochalasin B binding in the presence and absence of 500 mM glucose are shown in Fig. I.1. The results indicate that the binding of cytochalasin B is not displaceable by large quantity (500 mM) of D-glucose. The binding of L cells begin saturation at about 10 min. in the absence of glucose. However, the binding of cytochalasin B is still increasing after 20 min for the L cells when 500 mM glucose is present. These obervations imply that the glucose carrier of L cells cannot be estimated by cytochalasin B binding method.

CYTOCHALASIN B BINDING OF THYMOCYTES, SPLEEN CELLS AND PERITONEAL MACROPHAGES

Fig. I.2 shows the % bound of cytochalasin B versus different concentrations of cytochalasin B in spleen cells and thymocytes in the absence and in the presence of 500 mM glucose. The cytochalasin B binding sites in both cells seem to be not affected by the presence of 500 mM glucose. Thymocytes have only a half % bound when compared with spleen cells. The result of cytochalasin B binding experiment of peritoneal macrophages (Fig. I.3) gave similar % bound to that of spleen cells and was also glucose non-displaceable.

Fig. I.1 The time kinetics of cytochalasin B binding of L929 cells in the presence (○——○) and the absence (●——●) of 500 mM D-glucose.

The details of cytochalasin B binding are described in the text.

Values are presented as mean ± S.E.M. of quadruplicate determinations.

Fig. I.1

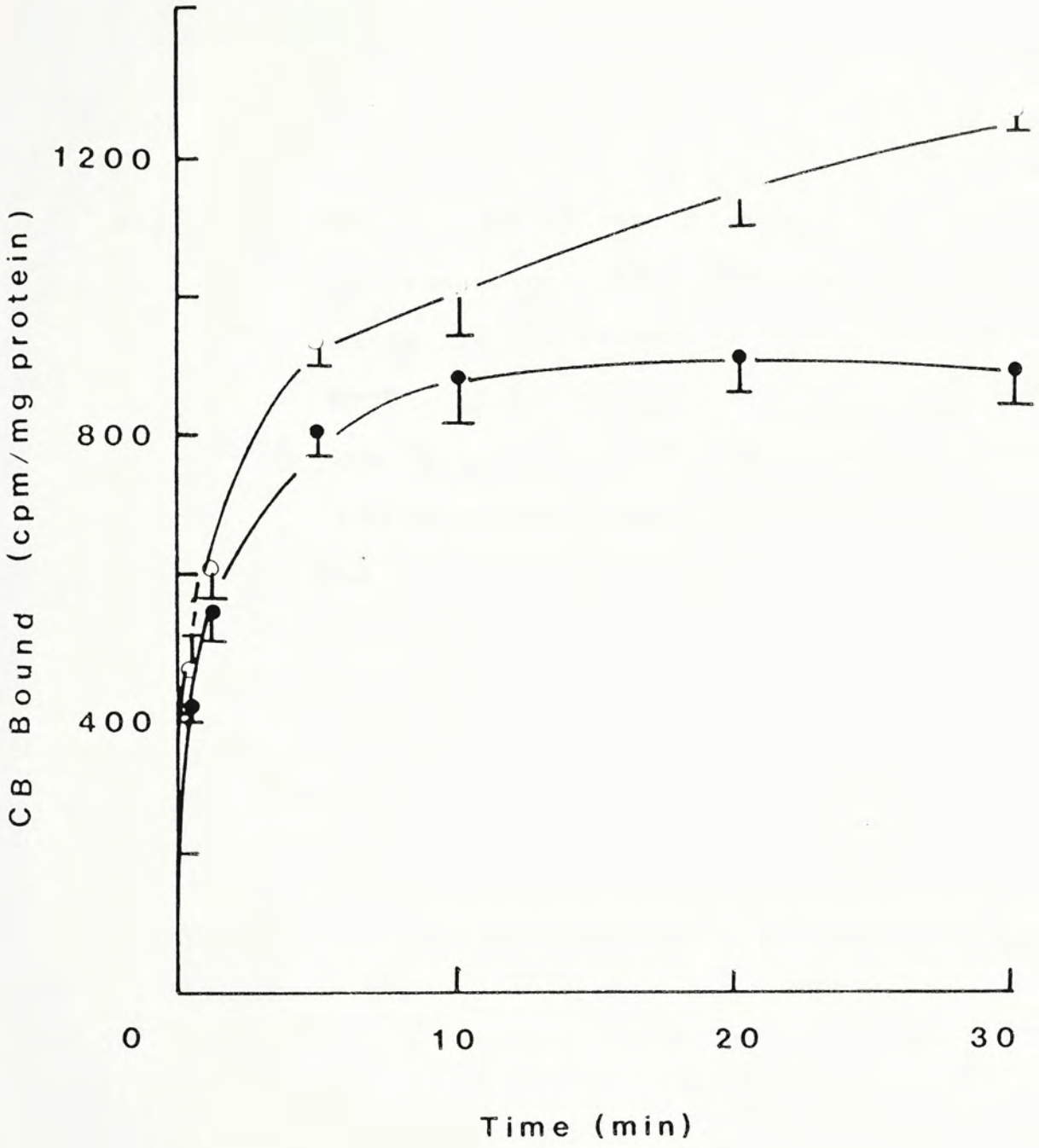


Fig. I.2 The % bound of cytochalasin B binding versus concentrations of cytochalasin B in spleen cells in the presence (○—○) and absence (●—●) of 500 mM glucose; and thymocytes in the presence (☆—☆) and absence (★—★) of 500 mM glucose respectively.

The detailed procedures for cytochalasin B binding are described in Chapter 3.

Fig. I.2

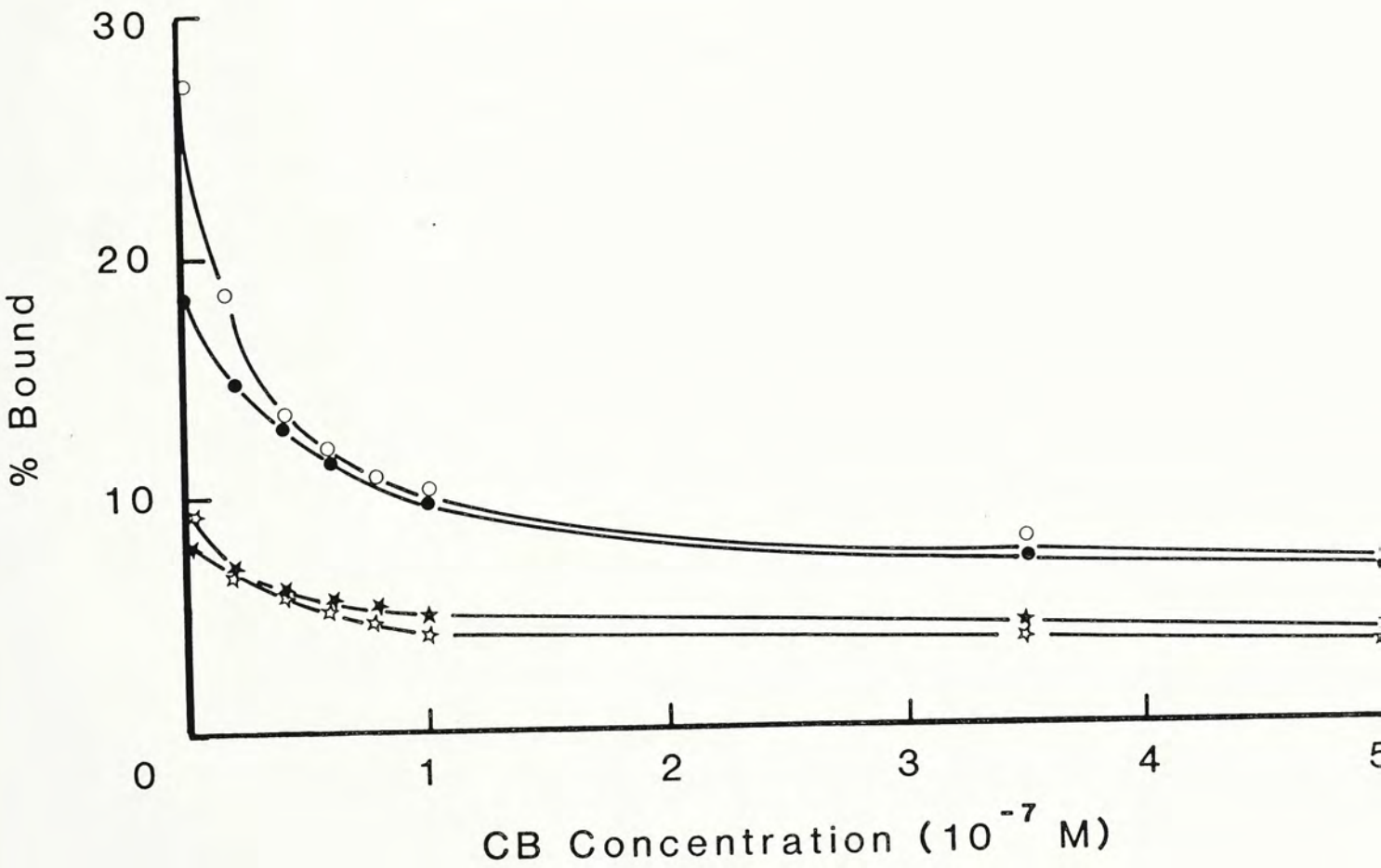
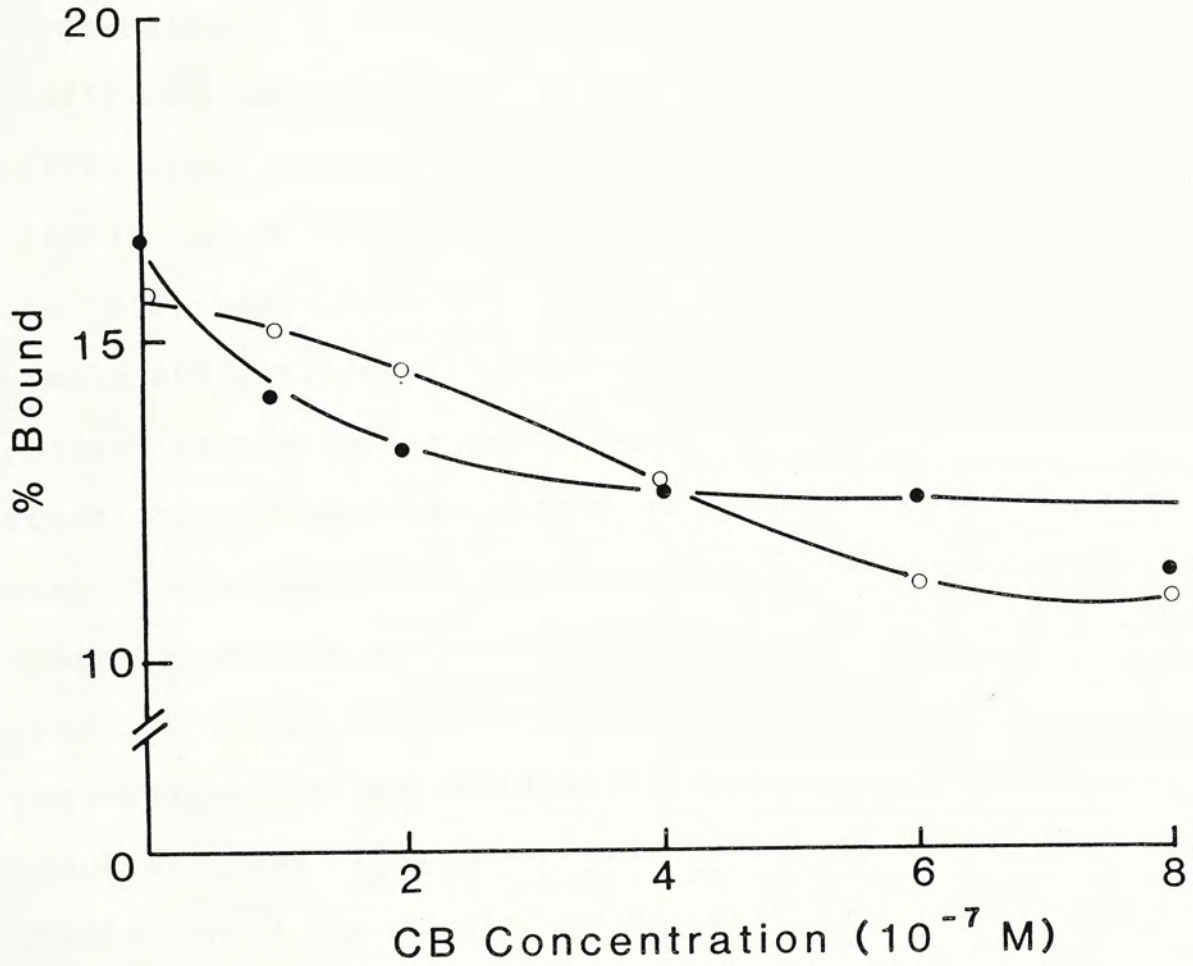


Fig. I.3 The % bound of cytochalasin B binding versus concentration of cytochalasin B in peritoneal macrophages in the presence (○ — ○) or absence (● — ●) of 500 mM glucose.
The detailed procedures for cytochalasin B binding are described in Chapter 3.

Fig. I.3



DISCUSSION

The present results indicate that cytochalasin B binding may not be displaceable by D-glucose in all cell types. The cytochalasin B binding in human erythrocytes (Jung & Rampal, 1977), transformed chicken embryo fibroblasts (Salter & Weber, 1979) and Ehrlich ascites tumor cells (Cuppoletti *et al.*, 1981), which can be easily displaced by glucose, thereafter, is different from the binding in L929, thymocytes, spleen cells and peritoneal macrophages. The cytochalasin B binding sites in the latter group may not necessarily be the glucose carrier although the uptake of 2-deoxy-D-glucose in L cells can be effectively inhibited by cytochalasin B (results unpublished in our laboratory). The binding site may be in the vicinity of glucose uptake site and the existence of cytochalasin B may sterically inhibit the uptake of 2-deoxy-D-glucose, and therefore the binding of cytochalasin B on L cells cannot be affected by the existence of glucose molecules (Fig. I.1). The lack of glucose-sensitive cytochalasin B binding in L cells, mouse thymocytes, spleen cells and peritoneal macrophages may show the fact that the glucose uptake properties and the nature of cytochalasin B binding are not universal for all kinds of cells. Similar findings were reported by Albert (1984) on rabbit erythrocytes and our laboratory on mouse erythrocytes (unpublished data).

APPENDIX II

EFFECT OF TUMOR NECROSIS SERUM ON MEMBRANE PERMEABILITY OF L929 CELLS

INTRODUCTION

When we studied the effect of tumor necrosis serum (TNS) on the glucose carrier of tumor cells, we found that TNS could reduce glucose uptake and density of glucose carrier in Ehrlich ascites tumor cells while enhancement of total glucose uptake was observed in TNS treated L929 cells (Chapter 6). Since the glucose carriers of L cells could not be estimated by cytochalasin B binding method (Appendix I), it is difficult for us to explain this paradoxical effect of TNS on different tumor cells. Since increase in total glucose uptake in TNS-treated L cells might be the result of increasing glucose simple diffusion or glucose transport mediated by glucose carrier, it is reasonable to study the change of cell membrane of L cells after TNS treatment. Furthermore, we are aware of no previous studies which examined the biochemical mechanism of action of TNS on cell membrane. The present study would give more detailed information for further understanding of the mechanism of antitumor effect of TNF.

EXPERIMENTAL

[^{3}H]-URIDINE UPTAKE OF L929 CELLS

The procedures for uridine uptake of L929 cells was similar to the procedures for 2-deoxy-D-glucose uptake of L929 cells (Chapter 3). 10^5 L cells, in 0.5 ml complete RPMI 1640 medium, were incubated with control serum or TNS for 24 hours at 37 °C. The monolayer of L929 cells then was washed twice with PBS and equilibrated to 37 °C. At time zero, 0.5 ml prewarmed [^{3}H]-uridine (100 uCi/umol) in PBS was added to give final concentrations of 25 - 100 uM. Reaction was stopped after 1 min by aspirating off the medium and rapid washing the cells twice with 1 ml ice-cold PBS supplemented with 0.05M uridine. The unsaturable diffusion was estimated by the presence of 20 uM (final concentration) of inhibitor dipyridamole or S-p-nitrobenzyl-6-thioguanosine (NBTGR) in the incubation medium. The cells were lysed by addition of 0.5 ml 0.1% Triton X-100 to individual well. Radioactivity of the lysate was counted in a Beckman LS-7000 liquid scintillation counter. Protein was determined by Lowry's method.

$^{45}Ca^{2+}$ UPTAKE

10^5 L929 cells, in 0.5 ml complete RPMI 1640 medium, were incubated with different dilutions of mouse sera at 37 °C for 24 hours. The monolayer was then washed twice with plain

RPMI. At various time intervals, 0.4 ml prewarmed $^{45}Ca^{2+}$-containing RPMI medium (0.7 mM, 0.076 uCi/well; 16.2 uCi/mM) was added to each well. Reaction was stopped by aspiration off the medium and rapid washing the cells three times with ice-cold saline containing 4 mM $LaCl_3$ to replace Ca^{2+} bound to the extracellular glycocalyx. Finally the cells were solubilized by adding 0.5 ml 1% Triton X-100. Radioactivity of the lysate was counted in a Beckman LS-7000 liquid scintillation counter. Protein was determined by Lowry's method.

[^{3}H]-METHIONINE UPTAKE AND ^{14}C-LEUCINE UPTAKE

Uptake of [^{3}H]-methionine (0.1 mM; 89 Ci/mmol) and ^{14}C-leucine (0.38 mM; 342 mCi/mmol) by L cells was carried out as for $^{45}Ca^{2+}$ uptake except that 0.02 uCi ^{14}C-leucine and 2 uCi [^{3}H]-leucine/0.4 ml RPMI medium was added to each well of culture plate. Reaction was stopped by using saline without $LaCl_3$.

RESULTS

EFFECT OF TNS ON URIDINE TRANSPORT

Fig. II.1 shows the results of a typical experiment of [^{3}H]-uridine by L929 cells *in vitro*. The rate of simple diffusion into the cells was obtained by simultaneous addition

of 20 uM dipyridamole or 20 uM NBTGR. Both dipyridamole and NBTGR can inhibit the facilitated uptake of [^{3}H]-uridine by L929 cells. The subtraction of uptake in the presence of inhibitor (i.e. unsaturable diffusion) from the total uptake in the absence of inhibitor will give the facilitated uptake of L cells (Fig. II.1B). The rate of facilitated uptake was rapid and linear in the first 1 min. The effect of TNS on the uridine uptake of L929 cells is shown in Fig. II.2. Cells treated with 1% TNS (v/v) have a significantly greater rate of simple diffusion than cells treated with control serum.

EFFECT OF TNS ON $^{45}Ca^{2+}$ UPTAKE

To investigate whether the action of TNF created an ionic imbalance within the target L929 cells, cellular uptake rate of $CaCl_2$ was determined. Fig. II.3 shows that addition of TNS significantly increased the $^{45}Ca^{2+}$ uptake rate by L929 cells.

EFFECT OF TNS ON AMINO ACIDS UPTAKE

To examine if TNS had a gross effect of membrane permeability of L929 cells, the ^{14}C-leucine and ^{3}H-methionine transport systems of TNF treated L929 cells were investigated. Fig. II.4 and Fig. II.5 show that neither ^{14}C-leucine nor ^{3}H-methionine uptake rates was altered in TNF treated L929 cells.

Fig. II.1 The time kinetics of [^{3}H]-uridine uptake of L929 cells incubated in RPMI medium.

II.1A [^{3}H]-uridine uptake in the absence (● — ●), or in the presence of 20 uM NBTGR (○ — ○), or 20 uM dipyridamole (☆—☆).

II.1B The facilitated uptake by subtracting the uptake in the presence of 20 dipyridamole from the total uptake in Fig. II.1A.

The values are presented as mean ± S.E.M. of quadruplicate determinations.

Fig. II.1A

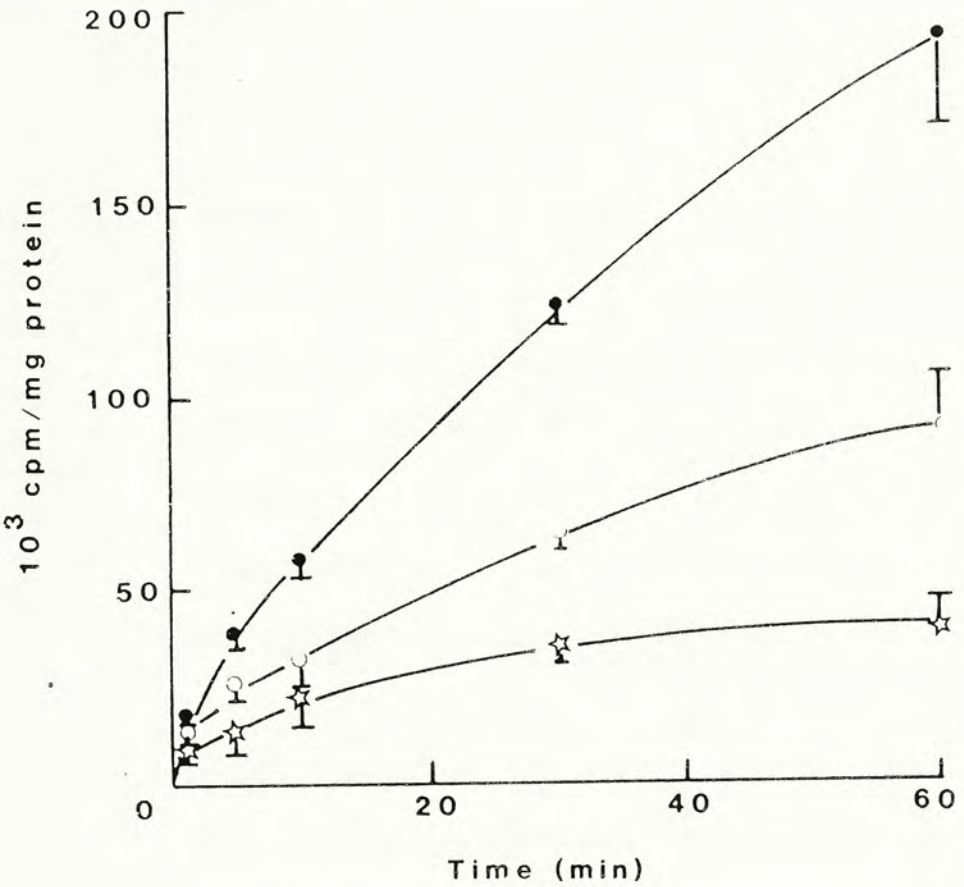


Fig. II.1B

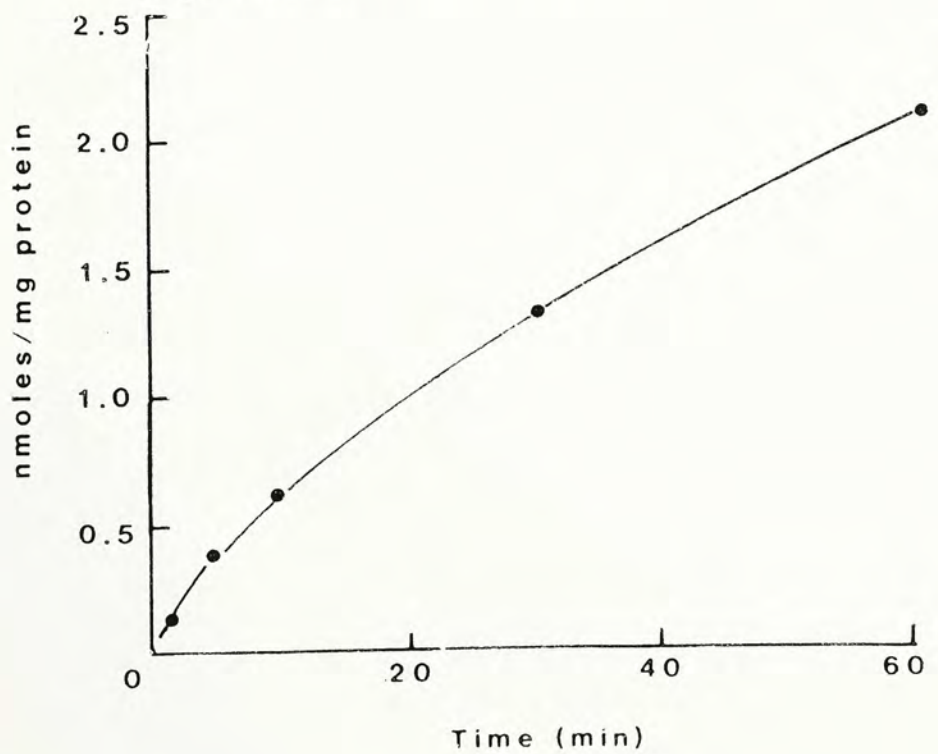


Fig. II.2 Effect of TNS on [^{3}H]-uridine uptake of L929 cells.

L929 cells, incubated with medium in the absence of (● — ●), or in the presence of (○ — ○) 20 uM (final concentration) dipyridamole; or incubated with medium supplemented with 1% (v/v) C. parvum-TNS for 24 hr in the absence of (▲ — ▲), or in the presence of 20 uM dipyridamole (△ — △).

The values are presented as mean ± S.E.M. of quadruplicated determinations.

Fig. II.2

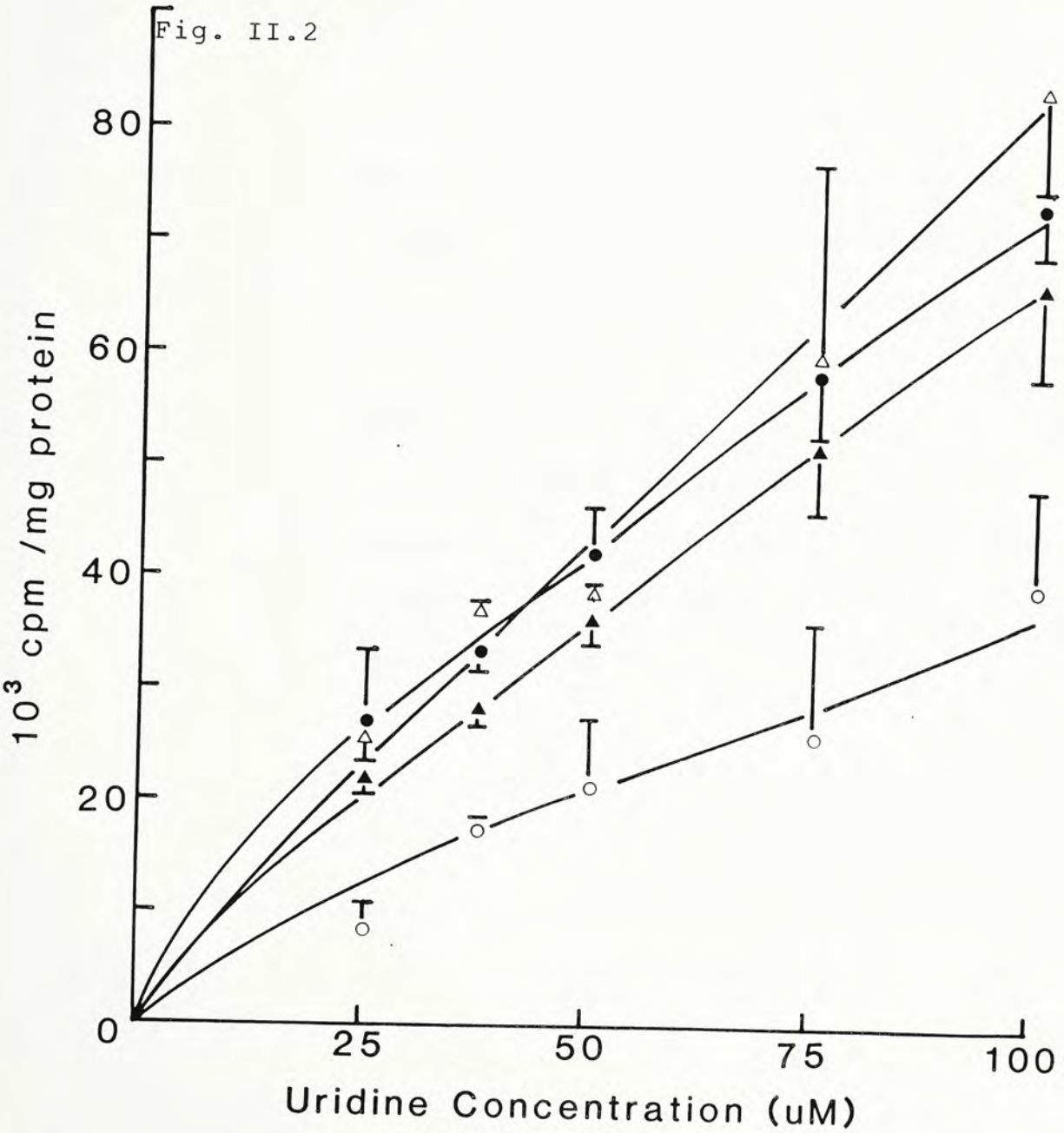

80
60
40
20
0
10^3 cpm /mg protein
25
50
75
100
Uridine Concentration (uM)

Fig. II.3 Time kinetics of $^{45}Ca^{2+}$ uptake of L929 cells incubated with media supplemented with 2% (v/v) control serum (●—●), or with 2% (v/v) C. parvum-TNS (○—○) for 24 hr before the uptake experiment.

The procedures of uptake are described in the text.

Values are presented as mean ± S.E.M. for quadruplicate determinations.

Fig. II.3

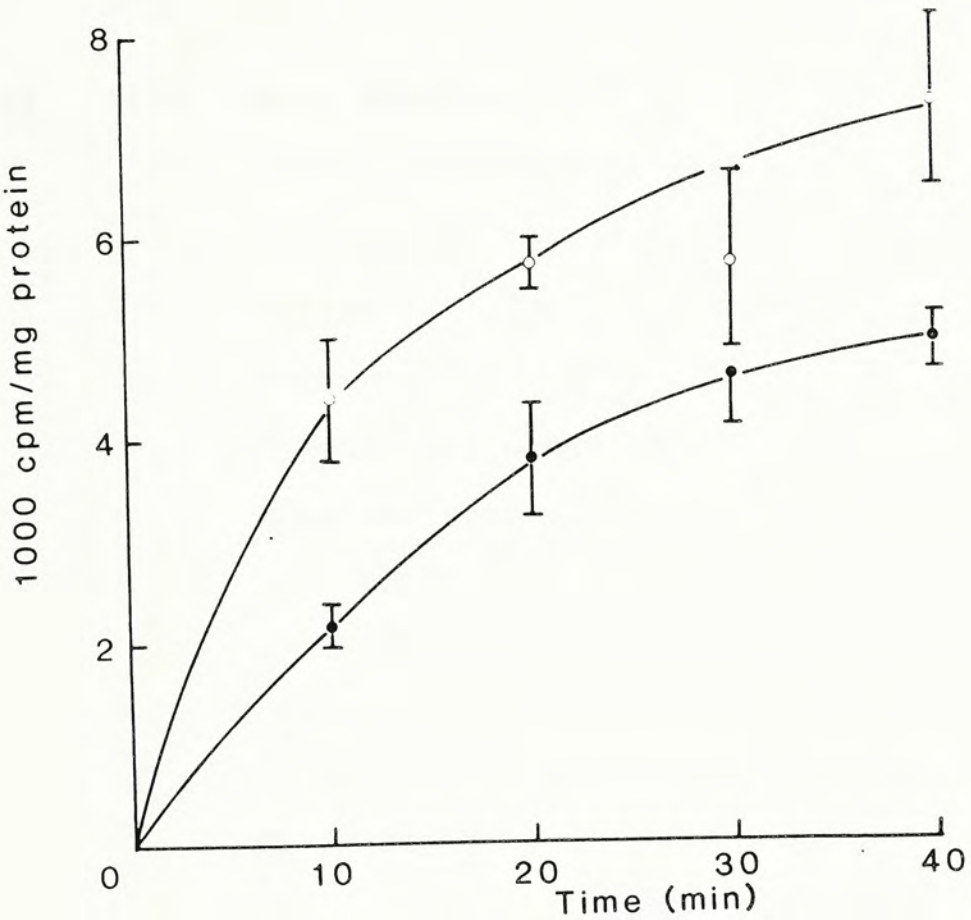


Fig. II.4 Time kinetics of ^{14}C-leucine uptake for L929 cells incubated in media supplemented with 2% control serum (●—●) or with 2% C. parvum-TNS for 24 hr before the uptake experiment (○—○).

Values are presented as mean ± S.E.M. for quadruplicate determinations.

Fig. II.4

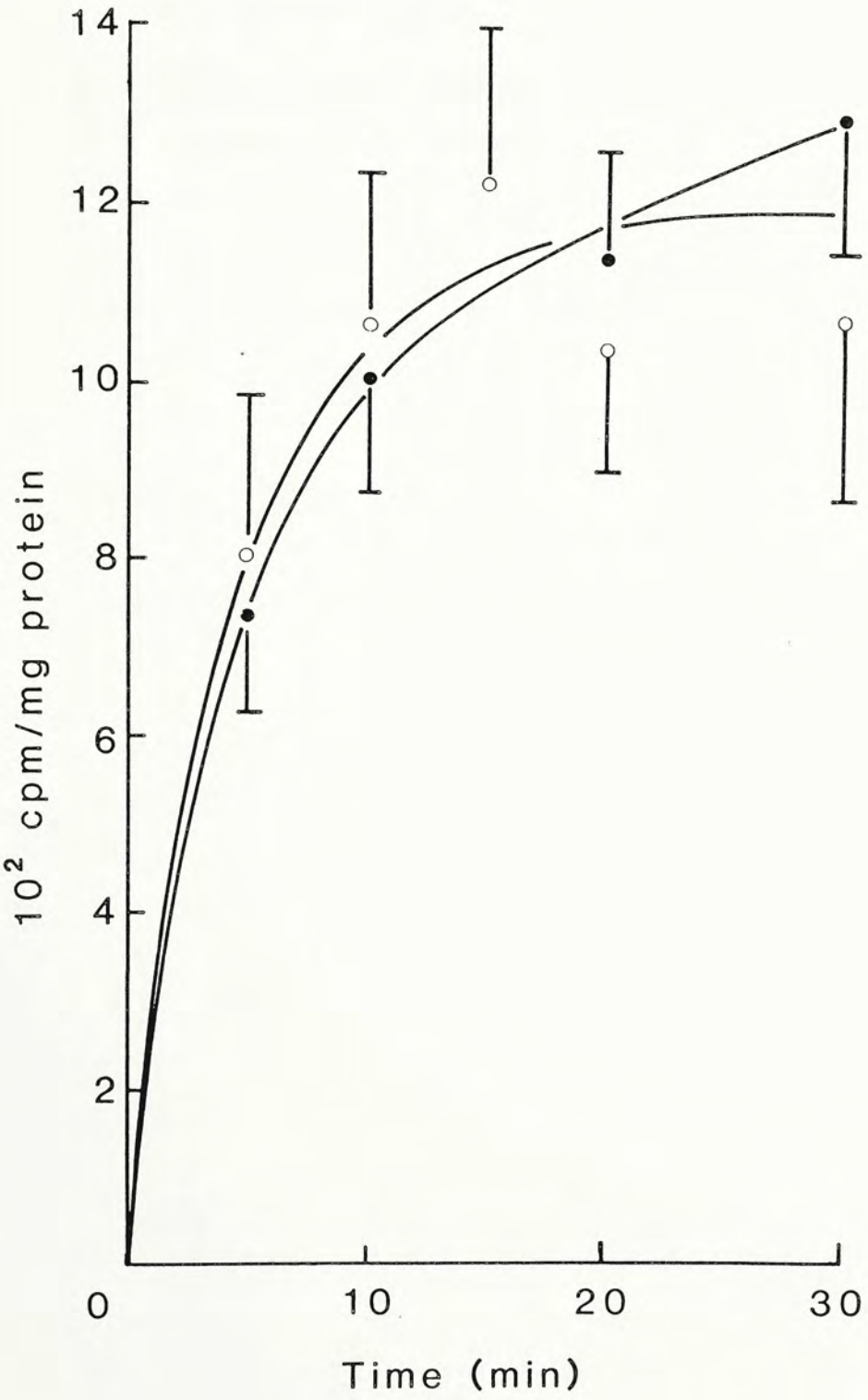
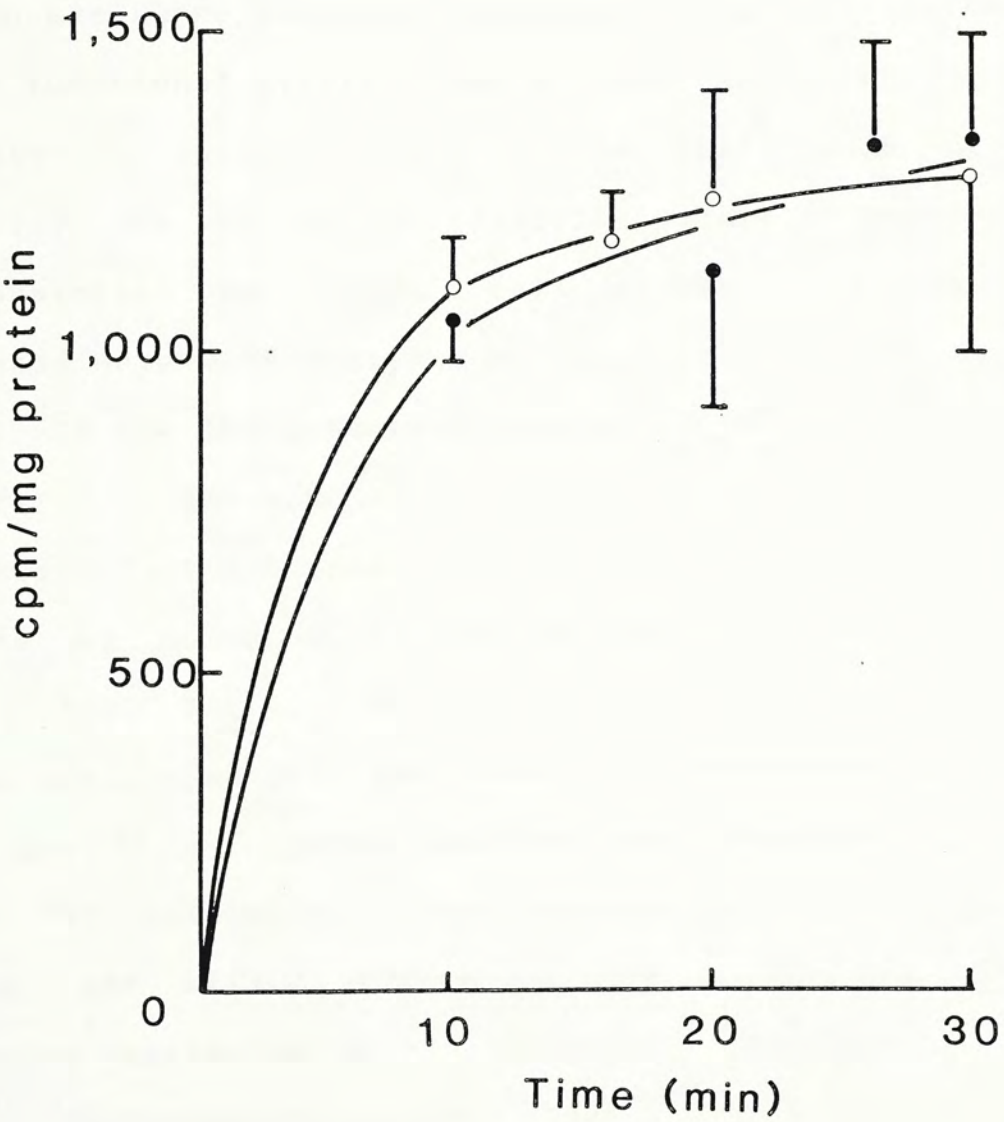


Fig. II.5 Time kinetics of [^{3}H]-methionine uptake of L929 cells incubated in media with 2% control serum (●—●) or with 2% C. parvum-TNS (○—○) for 24 hr. before the uptake experiment. Values are presented as mean ± S.E.M. for quadruplicate determinations.

Fig. II.5



Discussion

One of the basic questions concerning the mechanism of TNF-mediated cytolysis is whether TNF has a primary action site on the tumor cellular membrane, thus inducing structural and/or functional alternations of membrane, or whether the TNF molecule is incorporated into the cell, thus interupting primarily are of the intracellular metabolic pathways which are essential for normal cell growth. The experiments presented here were designed to explore, biochemically, the nature of the TNF-induced damage(s) to the plasma membrane of L929 cells. The results indicate that cells treated with TNS had a signifant increase in rate of simple diffusion of [^{3}H]-uridine as compared to that of cells treated with control serum (Fig. II.2). The active component of [^{3}H]-uridine uptake was, however, unaltered. Furthermore, we also found that the $^{45}Ca^{2+}$ uptake rate was also observed to increase after TNS treatment. This increase in $^{45}Ca^{2+}$ uptake rate during TNS action might indicate a failure of the Ca extruding mechanism or Ca influxing mechanism. Though we cannot differentiate between these two possible mechanism of TNF on L929 cells, we can draw a tentative conclusion that TNF might cause a change of permeability on membrane of L929 cells.

It is of interest to note that the total uptake rates

of two structurally related amino acid, leucine and methionine were not altered by TNS (Fig. II.3 and II.4). These findings suggest that TNS can selectively affect the rate of uptake of some biochemicals, for example, glucose, uridine and $^{45}Ca^{2+}$, and the other active transport systems are not necessarily affected. In this respect, lymphotoxin has been reported to have similar effect on L cells. Okamato and Mayer (1978) reported that Ca^{2+} uptake rate was increased while the K^{+} uptake rate, amino acid uptake rate (as determined by -aminoisobutyrate uptake) and dye exclusion ability were the same, in lymphotoxin treated L cells. Most recently, TNF and lymphotoxin are reported to be similar structurally (Gray et al., 1984). It is conceivable that these two antitumor agents might have similar actions on L cells.

However, data presenting in Chapter 6 and here still cannot indicate whether the TNS-induced changes of membrane permeability in L cells, resulting in enhanced uptake rate of certain biochemicals, is a primary or a secondary phenomenon. The TNS preparations used in these studies were still not purified. Therefore, there remains a possibility that the increase of the glucose, uridine and Ca^{2+} uptake rate in L929 cells might be induced by some contaminant. An answer to this question will be obtained only when highly purified preparations of TNF become available for study.

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